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# Studies of the chromosome of *Bacillus subtilis*

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STUDIES OF THE CHROMOSOME OF BACILLUS SUBTILIS

by

Judith Weaver Zyskind

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## INTRODUCTION

Today the bacterial chromosome is generally thought to be one circular double-stranded DNA helix containing the genetic information in a single linkage group and replicating sequentially in one direction from a fixed starting point. Several investigators, however, have hypothesized that the bacterial chromosome contains protein linkers. The initial object of this study was to develop ways of determining the existence or nonexistence of protein linkers in the Bacillus subtilis chromosome.

In the course of this investigation a question arose concerning the genetic transfer experiments of Sueoka and his coworkers (e.g., Yoshikawa and Sueoka, 1963b). Using the technique of transformation after density transfer experiments these investigators were able to demonstrate sequential DNA replication and to construct a genetic map of B. subtilis delineating an origin and terminus. One can ask why the newly labeled areas of the chromosome were found at the hybrid density in their experiments if whole chromosomes had been isolated. One answer could be that the replication point is a weak point in the chromosome where breakage occurs easily. Another answer could be that the labeled part of the chromosome is sufficiently heavy that it separates from the rest of the chromosome whether there is a replication point present in the chromosome or not. In order to examine this question experiments were designed to obtain a bacterial population which would contain chromosomes that were partly labeled with 5-bromouracil and that had no replication

points. If it is known which genetic markers are labeled, the fractionation in a CsCl density gradient of the DNA isolated from these cells should provide evidence for or against the weakness of the replication point.

## LITERATURE REVIEW

Because bacteria tend to stain uniformly with basic dyes, due to their rich cytoplasmic content of ribonucleic acid (RNA), it was generally believed either that bacteria constituted naked nuclei or else that they did not possess any form of discrete, organized nucleus. The development of cytochemical methods for the selective staining of deoxyribonucleic acid (DNA) eventually led to the clear demonstration, in all the bacterial species examined, of discrete chromatin bodies which could plausibly be interpreted as nuclear analogues (Robinow, 1944). Most of the information about the structure and organization of the chromatin bodies comes from electron microscopy of ultra-thin sections of bacteria. The chromatin body has the appearance of a finely fibrillar "nucleoplasm" which closely resembles that of the phage DNA pool (Kellenberger *et al.*, 1958; van Iterson and Robinow, 1961). These fibers frequently appear to be twisted about one another in parallel bundles, each bundle containing up to 500 fibers. Kellenberger (1960) proposed a tentative model of the way in which the DNA may be organized in the bacterial chromatin body. He suggested that the chromosome consists of about a thousand DNA fibrils which are joined end to end by small protein linkers to form a continuous, unbroken thread. The linkers are arranged together at the two poles of the body in such a way that the DNA fibrils zigzag back and forth between them to form a cylindrical surface. In view of the lack of direct evidence for protein or RNA linkers in the bacterial chromosome, Fuhs (1965) modified Kellenberger's model by removing the protein linkers, which re-



sulted in a model consisting of a bundle of DNA fibers folded back and forth. There are strong indications that chromatin bodies each contain the equivalent of only one bacterial chromosome (Schaecter et al., 1958; Young and Fitz-James, 1959).

"Chromosome" is a morphological term. Because there is little structural resemblance between the DNA-containing bacterial chromatin bodies and the chromosomes of eucaryotic organisms, Ris (1961) has suggested that the bacterial linkage group be called a "genophore" instead of a chromosome. The term "genophore" suggests only a genetic entity and, since the term "chromosome" also has definite genetic implications because in animal and plant cells the genophore is a chromosome, "bacterial chromosome" will be used in preference to genophore. When the bacterial chromosome is referred to strictly as a genetic unit, "genome" will be used. In this text genome indicates the genetic unit, excluding plasmids and episomes, which contains the main genetic information of the cell.

Genetic analyses of Escherichia coli show that all of the large number of genes now mapped are located on a single linkage group, the linkage data indicating a circular chromosome (Jacob and Wollman, 1958). Cairns (1963a and b), in his autoradiographic studies of labeled and carefully isolated DNA of E. coli, found continuous, unbranched DNA threads sometimes appearing as closed circles. His pictures suggest that the chromosome of E. coli consists of a single circular thread of double-stranded DNA 1100  $\mu$  long. Also, autoradiographic studies (Cairns, 1963b) as well as genetic studies (Yoshikawa and Sueoka, 1963a) of the replica-

tion of the bacterial chromosome show that replication is sequential from a fixed starting point of the chromosome, unidirectional to the end of the chromosome. Because of these experimental results the bacterial chromosome is generally thought to be one circular double-stranded DNA helix containing the genetic information in a single linkage group and replicating sequentially from a fixed starting point in one direction with one replication point. It is well established that the genes of E. coli are located on a single linkage group and that the linkage patterns provided by conjugation and recombination data can be explained only by a circular chromosomal structure. With respect to chromosomal replication, however, there are notable demonstrated exceptions. Oishi et al. (1964) observed the occurrence of dichotomous replication in Bacillus subtilis. They found that the chromosome in the spore is in completed form, and that, on germination in a minimal medium, the genes replicate in definite sequential order from one end of the chromosome to the other. In contrast, they found that germination in an enriched medium results in a dichotomous replication. The dichotomous replication observed in the enriched medium appeared to be a well regulated system in that re-initiation of replication of the chromosome is repressed until the first replication point reaches the middle region of the chromosome. Apparently, there are at least two replication points per chromosome, and, in density transfer experiments, Oishi et al. (1964) were able to estimate the positions of these two replication points on the chromosome of B. subtilis at different times. Dichotomous replication has also been reported in cells of E. coli

and B. subtilis when released from thymine deprivation (Pritchard and Lark, 1964; Kallenbach and Ma, 1968).

There is no definite or conclusive evidence that the bacterial chromosome consists of one long double-stranded DNA helix; also, there is no direct evidence for linkers in the chromosome. There are, however, strong suggestions that protein and/or RNA may be intimately associated with the structure of the bacterial chromosome. Spiegelman et al. (1959) isolated small vesicular bodies from protoplasts of Bacillus megaterium which contain virtually all of the protoplast DNA. These vesicular bodies resemble chromatin bodies morphologically, and, when purified and analyzed chemically, they are found to contain DNA, RNA, and protein in the ratio 1:1:3. Moreover, the protein fraction includes protamine- and histone-like substances in much higher concentration than protein derived from whole protoplasts. Several studies have confirmed that DNA synthesis depends on the synthesis of protein (Nakada, 1960; Maaløe and Hanawalt, 1961; Yoshikawa, 1965). It is likely that this protein mainly consists of enzymes required for DNA synthesis; but it is possible that it is also comprised of protein linkers which form an integral part of the chromosome structure.

Massie and Zimm (1965a), working with DNA from both B. subtilis and E. coli, have hypothesized that the bacterial chromosome is an assembly of DNA subunits of about 250,000,000 molecular weight held together by protein linkers. They offer three pieces of experimental evidence to support this hypothesis:

- 1) After cell lysis, the DNA could not be released from a heavy mass of protein-containing material until the protein had been removed with Pronase or phenol.
- 2) The approximate molecular weight of their DNA samples was consistently 250,000,000 daltons based on sedimentation velocity and intrinsic viscosity measurements.
- 3) There was little possibility of degradation in their DNA isolation procedure and yet the DNA molecules obtained by their procedure were smaller than the estimated molecular weight of the chromosomes of B. subtilis and E. coli by a factor of eight or more.

Most of the molecular weight estimates of the chromosome of B. subtilis are derived from chemical determinations of the amount of DNA per cell. Massie and Zimm (1965a) found that cultures of B. subtilis strain W23 in the stationary phase, with one to two nuclei per cell, contained from  $6.4 \times 10^{-9}$  to  $6.9 \times 10^{-9}$   $\mu$ g of DNA (equivalent to  $3.8 \times 10^9$  to  $4.2 \times 10^9$  daltons) per cell. Since B. subtilis strain W23, in the stationary phase, is reported to have chromosomes in the completed state (Yoshikawa and Sueoka, 1963a and b), the values of Massie and Zimm presumably would be for a single completed chromosome. Eberle and Lark (1967) estimated the amount of DNA per cell of B. subtilis at different growth rates. After correcting for the number of chromatin bodies per cell and the amount of replication per chromosome, they calculate the molecular weight to be  $3.0 \times 10^9$  to  $4.0 \times 10^9$  daltons for a nonreplicating,

completed unit.

The DNA of B. subtilis is double-helical (Mandell and Rowley, 1963) and, if it exists as a continuous piece in the hydrated form (B configuration, Langridge et al., 1957) having a mass per unit length of  $1.8 \times 10^6$  daltons per  $\mu$  (Langridge et al., 1960), it would have an over-all length of 2000  $\mu$  (based on a molecular weight of  $3.9 \times 10^9$  daltons). This value is more than twice the value reported for the length of the chromosome of B. subtilis estimated by autoradiography. Dennis and Wake (1966) in their autoradiographic study of the B. subtilis chromosome, found continuous chromosomal lengths of 800 to 900  $\mu$ . There are at least four possible explanations for this discrepancy. (1) Shearing of the chromosome could easily have occurred during the preparation of autoradiographs. (2) The use of detergent in lysing cells for the autoradiographs could have destroyed protein linkers. Cairns (1963b) found that completely intact replicating chromosomes from E. coli could be obtained only by using lysozyme without detergent. (3) It is possible that the chromosome of B. subtilis is composed of more than one replicon (independent replicating unit, Jacob et al., 1963). (4) There are two chromosomes per nucleus.

Recently, Yoshikawa (1968), from experiments with autoradiograms of germinating spores that had been uniformly labeled with  $^3\text{H}$ -thymidine, has shown that each B. subtilis spore contains two completed chromosomes. The DNA content of the B. subtilis spore sets an upper limit of  $3.0 \times 10^9$  daltons as the amount of DNA in its genome (Dennis and Wake, 1966). The value of  $3.0 \times 10^9$  to  $4.0 \times 10^9$  daltons calculated from growing cul-

tures (Eberle and Lark, 1967) is too large if each spore has 2 chromosomes. Therefore, the molecular weight of the chromosome of B. subtilis must be either  $1.5 \times 10^9$  or  $3.0 \times 10^9$  daltons, and, if the B. subtilis spore contains 2 chromosomes, the more correct value would be  $1.5 \times 10^9$  daltons.

The determination of the molecular weight of large DNA polymers (above  $1 \times 10^7$  daltons) has, until recently, been very difficult. Ample data on sedimentation coefficients and intrinsic viscosity of a large number of samples below  $1 \times 10^7$  daltons in molecular weight have been available for some time. Extensive data for DNA samples of higher mass are just becoming available; there are two reasons for this. In the first place, high molecular weight DNA samples were not available until it was recognized that shearing forces encountered in ordinary preparative procedures cause extensive degradation of DNA (Hershey and Burgi, 1960; Leventhal and Davidson, 1961). Second, measurements of the intrinsic viscosity and sedimentation coefficients for samples of high molecular weight posed difficult technical problems. The extrapolation to zero concentration required in the determination of the intrinsic viscosity had to be made from data obtained at a shear stress approaching zero. Zimm and Crothers (1962) claim this can be accomplished by using a low-shear viscometer which they designed. A serious anomaly related to rotor speed was encountered with high molecular weight samples during studies of sedimentation coefficients. It was found that the sedimentation coefficient can only be determined at low rotor speeds because,

at high rotor speeds, a variable fraction of the high molecular weight DNA behaves like a precipitate and rapidly sediments out of solution (Rosenbloom and Schumaker, 1967).

Intrinsic viscosity is a property which can be used to estimate the molecular weight of semi-large to large molecules such as proteins, RNA's, and DNA's. It is a measurement of the force with which molecules resist movement when exposed to an outside force. The viscosity of high molecular weight DNA is highly dependent both upon shear stress and concentration. Crothers and Zimm (1965) have been able to demonstrate with their low-shear viscometer that, at very low shear stresses, the viscosity is virtually independent of shear stress. There appears to be no explanation in the literature suggesting why the viscosity of high molecular weight DNA has such a strong dependence on shear stress. It is unlikely that shear stresses exerted by capillary viscometers are strong enough to break the DNA molecules. It was suggested that during the measurement of viscosity the DNA molecules become aligned so that the force of resistance offered by these molecules is mainly due to the diameter of the double helix (personal communication, Michael Burke, Biochemistry and Biophysics Department, Iowa State University, Ames, Iowa).

The sedimentation coefficient,  $S_{w,20}^0$ , is also a parameter used to estimate molecular weight. For most molecules, the sedimentation coefficients are independent of rotor speed. This is not true for DNA of a molecular weight above  $1 \times 10^7$ . Several investigators found that, as the rotor speed was raised, the sedimentation coefficient increased.

Also, they noticed that a fraction of the DNA appeared at the bottom of the cell. Rosenbloom and Schumaker (1967) have studied this phenomenon carefully. They suggested that the collision frequency of DNA molecules is increased at high rotor speeds, and that the colliding DNA molecules form dimers, trimers, and larger aggregates. This ultimately leads to the separation of a solid phase which precipitates out of solution and sediments rapidly to the bottom of the cell. The sedimentation coefficient is then dependent upon the concentration of the DNA remaining in solution. These anomalous effects can be avoided by using rotor speeds below 15,000 rpm (Aten and Cohen, 1965).

Transformation was first observed in Diplococcus pneumoniae (Griffith, 1928; Avery et al., 1944). It can be defined as the uptake by recipient bacteria of DNA extracted from donor bacteria and the subsequent incorporation and expression of the donor DNA in the recipient. As transformation studies were extended, it was found that this phenomenon occurs regularly in other genera. Also, a number of cases of interspecific and even intergeneric transformations have been reported (Schaeffer, 1963). The discovery by Spizizen (1958) that the transformation reaction occurs in B. subtilis has been exploited by several workers.

The genetic markers in B. subtilis so far studied have not, thus far, been established as being associated with a single replicon (Dubnau et al., 1967), although many of them occur as linked clusters and most appear to replicate in a sequential order (Yoshikawa and Sueoka, 1963a and b; Oishi et al., 1964; O'Sullivan and Sueoka, 1967; Dubnau et al., 1967). The replication order of several markers in the chromosome of B. subtilis



was first determined by marker frequency analysis using transformation (Yoshikawa and Sueoka, 1963a). A theoretical treatment of marker frequency analysis is given by Sueoka and Yoshikawa (1965). Their theory is based on the replication model in which the chromosome replicates from one end (origin) toward the other end (terminus) at an approximately uniform speed with both strands replicating. With this model the frequency of a marker in an exponentially growing cell population is directly related to the position of the marker on the chromosome. Sueoka (1966) has summarized the data from marker frequency analyses in a map (Figure 1B). The order of markers in this map is in general agreement with the order of markers as determined by density transfer experiments (Figure 1A). O'Sullivan and Sueoka (1967) have performed two types of density transfer experiments, both utilizing the synchronization of chromosome replication during spore germination (Oishi et al., 1964). In one type of experiment, deuterium oxide-grown spores were allowed to germinate in a rich aqueous medium and, in the other type of experiment, spores of a thymine-requiring strain were allowed to germinate in a medium containing 5-bromouracil. Samples were taken at various times during germination and lysates, prepared from the samples, were centrifuged in a density gradient. The transfer of genetic markers from non-replicated, half-replicated, and fully replicated DNA was followed by transformation assays. They found that although synchrony deteriorated less rapidly in D<sub>2</sub>O transfer experiments than in 5-bromouracil transfer experiments, the density differences obtained for light, hybrid, and heavy DNA were much greater with 5-

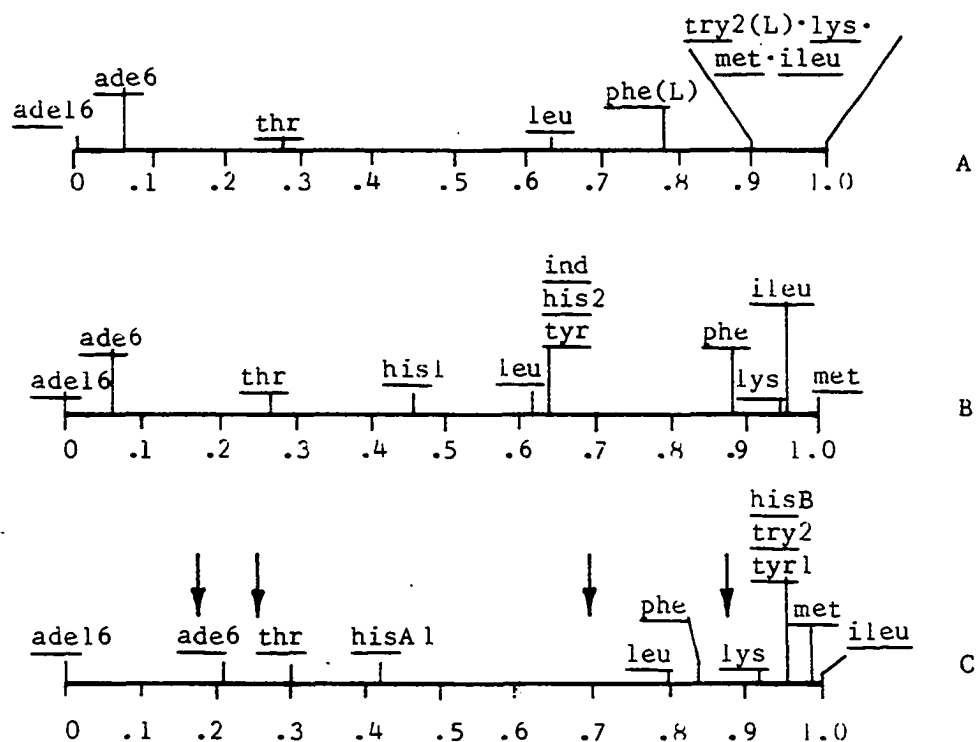


Figure 1. Comparison of genetic maps of *B. subtilis*. The maps were obtained by density transfer analysis, marker-frequency analysis, and a combination of transduction data and density transfer analysis. Map A is based on density transfer data using primarily strain 168. (from O'Sullivan and Sueoka, 1967). Map B is constructed from marker-frequency analyses with strain W23 as summarized by Sueoka (1966). Map C was constructed by Dubnau *et al.* (1967) from genetic linkage data obtained using transduction in strain 168. The linkage groups were located and oriented by Dubnau *et al.* from the results of density transfer experiments carried out in strain W23. The linkage gaps are marked with vertical arrows. Dubnau *et al.* obtained several of Sueoka's mutants so that markers adel6, ade6, thr, leu, phe, lys, met, and ileu are identical in all three maps. The his1, his2, ind, and tyr markers of Sueoka (1966) are assumed to be homologous to hisA1, hisB, try2 and try1 mutations of Dubnau *et al.* (1967).

bromouracil. Because synchrony deteriorates during the latter period of replication this method is only useful for markers in the early replicating part of the chromosome; thus, O'Sullivan and Sueoka did not assign any map positions to the markers try 2(L), met, and ileu (Figure 1A).

Dubnau et al. (1967) constructed a map of the genome of B. subtilis from the results of density transfer experiments and from genetic linkage data obtained using DNA-mediated transformation and phage-mediated transduction (Figure 1C). Only two major differences exist between their map and the map (Figure 1B) of Sueoka (1966). One difference is the reversal of the markers met and ileu. Because the use of transduction linkage data gives a more precise analysis of closely linked markers than density transfer or marker-frequency analysis, the map in Figure 1C contains the most likely order for the markers met and ileu. This would also hold true for the second discrepancy involving the aromatic amino acid cluster, ind, his2, and tyr. Dubnau et al. (1967) found the aromatic amino acid cluster linked to lys, met and ileu but not to leu. Kelly and Pritchard (1965) also found a similar linkage pattern. It is therefore likely that the map in Figure 1C indicates the correct position of the aromatic amino acid cluster.

Treick and Konetzka (1964) studied the effects of bacteriostatic concentrations of phenethyl alcohol (PEA) on the synthesis of DNA, RNA, and protein by cultures of E. coli. They found that DNA synthesis by cells of E. coli from the maximum stationary phase was completely inhibited by 0.32% PEA immediately upon the addition of the inhibitor,

although there was a net increase in the synthesis of RNA and protein. However, DNA synthesis in cells from the exponential phase was inhibited only after an increase which corresponds to 1.4 to 1.6 times the amount of DNA present at the time of PEA addition. By programming the addition and removal of PEA, Treick and Konetzka (1964) found that DNA synthesis proceeded in stepwise increments corresponding to doublings of the DNA. They suggested that the DNA being replicated at the time of the PEA addition completes the replication cycle and no initiation of a second cycle of DNA replication occurs until the removal of PEA. In other investigations of the mode of action, PEA has been found to inhibit the process of enzyme induction (Rosenkranz et al., 1965), inhibit the development of spore septum and membranes (Remsen et al., 1966), cause a breakdown of the cellular permeability barrier (Silver and Wendt, 1967), inhibit the growth of an RNA phage (Nonoyama and Ikeda, 1964), and inhibit the germination of spores (Lester, 1965; Slepecky, 1963). Because most of these actions are thought to be associated with the cell membrane, Silver and Wendt (1967) proposed that the primary effect of PEA is a limited breakdown of the membrane.

In 1955, Lerman introduced the use of columns composed of methylated albumin absorbed on Kieselguhr (MAK) for the fractionation of DNA. Mandell and Hershey (1960) developed this column to such an extent that they were able to separate several ribonucleic acids and deoxyribonucleic acids from each other, including the nucleic acids of T2 and T4 bacteriophages. Saito and Masamune (1964) attempted to fractionate DNA of B. subtilis with a MAK column prepared according to Mandell and Hershey (1960). When a

linear gradient of NaCl between 0.64 M and 0.80 M was applied to the column, approximately 40% of the applied DNA was recovered in fractions eluted with 0.66 M NaCl. The fractions were examined for their ability to transform recipient strains. Based on this transformation data, Saito and Masamune (1964) claimed that certain genetic marker activities were enriched in restricted fractions.

## MATERIALS AND METHODS

## Media and Buffers

The media used in this investigation include brain heart infusion (BHI, Difco) agar, trypticase soy agar (TSA, Baltimore Biological Company), Staphylococcus synthetic medium (Weaver and Pattee, 1964), E medium (Vogel and Bonner, 1956), Minimal Medium (Table 1), Medium S (Table 2), Medium T (Table 3), Minimal Glucose Agar (Minimal Medium supplemented with 0.5% glucose; 1.5% Noble's agar, Difco; 0.001% required amino acids; and 0.005% adenine, if required), Sporulation Agar (Oishi and Sueoka, 1965), Schaeffer's Sporulation Medium (Yoshikawa, 1965), and Germination Medium (Table 4). Medium S and Medium T are the same as media described by Anagnostopoulos and Spizizen (1961) as modified by Oishi et al. (1964), except that in Medium T the required amino acids were added at 1/10 the concentration suggested by Oishi et al. The basal salts in the Minimal Medium were prepared at 5 times the final concentration used. Separate stock solutions of the amino acids and thymidine (TdR, Calbiochem, Los Angeles, California) were prepared at a concentration of 10 mg per ml. The stock solutions of adenine and 5-bromodeoxyuridine (BUdR, Calbiochem) were prepared at 5 mg per ml. All media were autoclaved for 15 min at 121 C. Glucose was autoclaved separately as a 50% solution and added aseptically to sterile media. All agar plates were dried overnight at 37 C prior to use.

The BPES buffer of Crothers and Zimm (1965), consisting of 0.006 M  $\text{Na}_2\text{HPO}_4$ , 0.002 M  $\text{NaH}_2\text{PO}_4$ , 0.001 M disodium dihydrogen ethylenediamine-tetraacetic acid (EDTA), and either 0.1 M NaCl (0.1 M BPES) or 1.0 M

Table 1. Composition of Minimal Medium

Constituent	Amount
K <sub>2</sub> HPO <sub>4</sub>	14.0 gm
KH <sub>2</sub> PO <sub>4</sub>	6.0 gm
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.0 gm
Trisodium citrate · 2H <sub>2</sub> O	1.0 gm
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.2 gm
Deionized water	1000.0 ml

Table 2. Composition of Medium S

Constituent	Amount
Minimal Medium	1000.0 ml
Casamino acids (Difco)	500.0 mg
L-Tryptophan	50.0 mg
Required amino acids, each	100.0 mg
Adenine, if required	50.0 mg
Glucose	5.0 gm

Table 3. Composition of Medium T

Constituent	Amount
Minimal Medium	1000.0 ml
Casamino acids	100.0 mg
L-Tryptophan	5.0 mg
L-Histidine	5.0 mg
Required amino acids, each	5.0 mg <sup>a</sup>
Adenine, if required	5.0 mg
Glucose	5.0 gm

<sup>a</sup>One-tenth the concentration suggested by Oishi et al. (1964).

Table 4. Composition of Germination Medium

Constituent	Amount
Minimal Medium	1000.0 ml
L-Alanine	100.0 mg
L-Glutamic acid	100.0 mg
Casamino acids	500.0 mg
Yeast extract (Difco)	200.0 mg
Required amino acids, each	100.0 mg
Glucose	5.0 gm

NaCl (1.0 M BPES), was used in the DNA isolation procedure. This buffer is effective in inhibiting nuclease activity (Massie and Zimm, 1965).

#### Chemicals

Methylated albumin, ribonuclease (RNase), deoxyribonuclease (DNase), and calf thymus DNA were purchased from Worthington Biochemical Corporation, Freehold, New Jersey, while lysozyme and Pronase were obtained from Calbiochem, Los Angeles, California. Phenethyl alcohol (PEA) was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin.

The stock solution of RNase which contained 1 mg RNase per ml of 0.1 M BPES buffer was boiled for 10 min to inactivate any contaminating DNase in the preparation.

A final concentration of 20  $\mu$ g DNase per ml was used to terminate the transformation reaction. The stock solution was prepared by dissolving an 11-mg sterile sample of DNase in 45 ml and then adding 5 ml of a solution containing 100 mg  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per ml and 5 ml of a 1.0 M Na



acetate solution. When this stock containing 200 µg DNase per ml is diluted 1:10 into Medium T, the final concentration of  $\text{MgSO}_4$  is 0.005 M, the concentration for optimum activity of DNase.

A Pronase solution was prepared in 0.1 M BPES buffer as described by Hotta and Bassel (1965). "A stock solution of the protease, Pronase (Calbiochem) was prepared at a concentration of 2 mg/ml. The pH was adjusted to 5.0 with HCl, heated to 80 C for 10 min, cooled, the pH re-adjusted to 7.0 with NaOH, and solid NaCl added to a concentration of 1.0 M. The solution thus prepared was DNase-free and could be stored for at least several months at -20 C without loss in activity."

The stock solution of lysozyme containing 50 mg lysozyme per ml of 0.1 M BPES buffer was prepared and filter sterilized just prior to use. PEA was also filter sterilized.

#### Bacterial Strains

All of the strains of Bacillus subtilis employed in this study were obtained from Noboru Sueoka, Department of Biology, Princeton University, Princeton, New Jersey. Donor DNA for transformation was prepared from strains W23 and 23 thy-his. Strain 23 thy-his is a double auxotrophic mutant of strain W23 which is dependent on thymine and histidine for growth. Strains W23 and 23 thy-his were used as sources of DNA because, according to Oishi et al. (1964), strain W23 has a completed chromosome in the stationary phase while strain W168 has a completed chromosome only in spores, and, at present, high molecular weight DNA can be isolated

from cells, but not from spores. Because mutants of strain W23 are not transformable, and mutants of strain W168 are, strains 168 leu-met-ade16 and 168 leu-met-thr (Sueoka's Mu8u5u16 and Mu8u5a5, respectively) were used as recipients in the transformation studies. Strains 168 leu-met-ade16 and 168 leu-met-thr are triple auxotrophic mutants of strain W168. Strain 168 leu-met-ade16 requires leucine, methionine, and adenine for growth, while strain 168 leu-met-thr requires leucine, methionine, and threonine for growth.

The stock cultures were maintained on TSA slants in screw-capped tubes except for strain 23 thy-his which was maintained on TSA slants containing 25 ug of thymine per ml. After inoculation, the slants were incubated for 3 days at 37 C, then stored at 4 C. Under these conditions the stock cultures remained viable and were used as the source for preparing subcultures for the duration of this investigation.

Staphylococcus aureus strain 655 (Pattee and Baldwin, 1961) and Escherichia coli B were used in the studies of the effects of PEA.

#### Isolation of High Molecular Weight DNA

##### Pronase procedure

The method of Massie and Zimm (1965a) is cited. "At 37 C, 500-ml cultures of E. coli and B. subtilis 168 were grown to the beginning of stationary phase and cultures of B. subtilis W23 were grown 4 hr into stationary phase. The cells were then centrifuged down at 0 C and re-suspended in 50 ml of cold BPES at pH 9. For coli it was then necessary

to heat for 20 min at 70 C to inactivate nucleases; omission of this step yielded DNA with molecular weight below  $9 \times 10^7$ . In the case of subtilis, heating had no effect. Lysozyme was added to 5 mg/ml and the suspension poured into a specially treated dialysis bag where it was left for the rest of the isolation process. The dialysis bag had been treated with 64%  $\text{ZnCl}_2$  for 15 min in order to increase its porosity to the point where oligopeptides and oligonucleotides would pass through freely. The bag (18 mm in diameter) was suspended in a vertical glass cylinder 40 cm long, the bottom of which was immersed in a water bath. Lysozyme treatment was continued for about 30 min at 37 C, the molecular weight of the DNA finally obtained being independent of the time of treatment. The temperature was then raised to 50 C and a Pronase solution, prepared as described by Hotta and Bassel, was added to a final Pronase concentration of 1 mg/ml. Pronase was replenished by the addition of 0.5 mg/ml every 4 hr. Fresh BPES buffer was caused to flow around the bag at about 25 ml/hr; convection from the heat of the bath caused sufficient stirring without the danger of shear. Pronase treatment was normally continued for at least 24 hr until a sample of the lysate gave no white cloudy precipitate when centrifuged at 10,000 g for 10 min. At this point RNase solution (previously boiled to destroy DNase traces) was added to a final enzyme concentration of 1 mg/ml and incubated 3 hr at 50 C. Pronase treatment was then resumed to remove the RNase. If RNA was still present (as shown by excessive UV absorption), the procedure was repeated. When desired, the last traces of protein were removed by

the phenol method (see below). The yield by this method was greater than 50% of the initial DNA."

#### Hot phenol procedure

The method of Massie and Zimm (1965b) is cited. "The DNA-containing material is suspended in a "1-molar" pH 7 buffer (NaCl, 1 M; EDTA, 0.001 M;  $\text{NaH}_2\text{PO}_4$ , 0.002 M;  $\text{Na}_2\text{HPO}_4$ , 0.006 M) and warmed to 55 C in a glass centrifuge tube in a water bath. An equal volume of buffer-saturated phenol is then added dropwise and the mixture allowed to stand at 55 C for about 5 min. At this temperature the phenol phase has practically the same density as the salt solution, so that phenol drops remain suspended throughout the solution and all parts of the solution come in close contact with the phenol without the need of shaking. The solution is then chilled and centrifuged cold to remove the suspension of fine reprecipitated phenol and denatured protein, if any. The phenol treatment may be repeated as many times as desired. Finally, the supernatant is transferred with a wide-mouthed pipette and the dissolved phenol is dialyzed away."

#### Preparation of Spores

The growth from 4 TSA slant cultures grown for 16 hr at 37 C was inoculated into 4 Fernbach flasks each containing 1 L of Schaeffer's Sporulation Medium. The flasks were shaken on a New Brunswick Model VL platform shaker at a setting of "1" at 37 C. After 5 days the mixture of cells and spores was centrifuged at 16,000 X g at 4 C using a Servall

RC-2B centrifuge with a GSA head and resuspended in 400 ml of sterile deionized water. This mixture was refrigerated overnight at 4 C, then centrifuged as before and resuspended in a 200 ml filter sterilized solution of 0.01 M tris - 0.001 M EDTA buffer, pH 8.4, containing 1 mg of lysozyme per ml. After incubation for 1 hr at 37 C lysis of cells was completed by addition of 2% sodium lauryl sulfate and incubation at 37 C for 30 min. The spores were centrifuged and washed 4 times in 500 ml of sterile deionized water. The purified spores were resuspended in 20 ml sterile deionized water and kept at 4 C. This method yielded approximately  $1 \times 10^{12}$  spores or  $5 \times 10^{10}$  spores per ml in the stock spore suspension.

#### Spore Counts

The number of spores was estimated from the average of 3 separate counts made in a Petroff-Hausser counting chamber.

#### Spore Germination Studies

Germinating spore suspensions were mixed 1:1 with 0.6% cetyl pyridinium chloride to improve the resolution of cell walls (Neville and Holt, 1968) and examined microscopically under dark high phase contrast using an American Optical Company Phasestar microscope and an Ortho-Illuminator. The time of the first division was taken as that time when a single cell septum could be seen in over 50% of germinated spores. The time of the second division was determined from the time three septa per cell could be seen in over 30% of the germinated spores.

### Transformation

Transforming activities were assayed according to a modified method of Anagnostopoulos and Spizizen (1961). Competent recipient cells were prepared as follows. Two ml of a mutant cell suspension grown overnight in Medium S were diluted in 40 ml of the same medium in a 250 ml side arm flask. The culture was shaken on a New Brunswick Model VL platform shaker at a setting of "1" at 37 C, and the optical density (O.D.) at 535 m $\mu$  was recorded at 1 hr intervals. When growth approached the stationary phase, this cell suspension was diluted 1:10 into Medium T which had been prewarmed. After 90 min incubation at 37 C with shaking, the cells were considered competent. Repeated experiments have shown that optimum competency is generally achieved after 90 min of shaking. In measuring transforming activities 0.9 ml of the competent cell suspension was added to 0.1 ml of a DNA sample and incubated for 40 min at 37 C with constant shaking on a Model 2156 Research Specialties Co. shaker at a setting of "10". DNase (20  $\mu$ g/ml) was then incubated with the transformation mixture for 15 min in order to stop the reaction. Generally, 0.1 ml of the transformation mixture or a dilution of the transformation mixture was spread by means of a bent glass rod on the surface of Minimal Glucose Agar plates. Duplicate plates were spread for each dilution. After 24 hr incubation at 37 C the number of colonies was counted. The Minimal Glucose Agar plates were enriched with casamino acids to speed the growth of the colonies. When thr and ade transformants were selected, the concentration of casamino acids added was 0.005%. When leu and met

transformants were selected, the casamino acids concentration added was 0.001%.

The results were generally recorded either as frequency of transformation (%), relative marker frequency, or relative no. of transformants (%).

$$\text{Frequency of transformation (\%)} = \frac{\text{transformants/ml of transformation reaction mixture}}{\text{total cells/ml of transformation reaction mixture}} \times 100$$

$$\text{Relative marker frequency} = \frac{\begin{array}{l} \text{no. of marker} \\ \text{"X" transformants/no. of met} \\ \text{transformants} \end{array} \text{ in DNA sample}}{\begin{array}{l} \text{no. of marker} \\ \text{"X" transformants/no. of met} \\ \text{transformants} \end{array} \text{ in DNA isolated from a stationary phase culture}}$$

$$\text{Relative no. of transformants (\%)} = \frac{\text{transformants/ml of fraction from CsCl density gradient centrifugation}}{\text{transformants/ml of control DNA}} \times 100$$

The frequency of transformation indicates the percentage of cells competent for a particular marker in the total cell population. This varies from marker to marker even when using DNA from a stationary culture of B. subtilis strain W23 where most of the cells should have a complete chromosome and all the markers should be equally frequent. This is most likely because of the differences in the integration efficiencies of the different markers (Sueoka and Yoshikawa, 1963). Therefore, mere comparisons of numbers of transformants for different markers do not indicate the actual frequencies of the markers in the DNA preparation in question. To solve this problem, Sueoka and Yoshikawa (1963), for each marker and

each DNA sample, normalized the number of transformants obtained for a particular marker to the number obtained for the met marker. Then these normalized values were normalized to the value for a DNA sample isolated from a stationary phase culture. These final values are referred to as relative marker frequencies.

The relative no. of transformants is an indication of the amount of DNA coding for a particular marker in a sample fraction from a CsCl density gradient centrifugation. The control DNA used was a sample removed from the DNA sample to be fractionated prior to the addition of CsCl.

#### Viable Cell Counts

The number of viable cells in a cell suspension was determined by preparing 2 serial 10-fold dilution sets of the suspension in 4.5 ml of Minimal Medium. Two TSA plates were spread with 0.1 ml from each dilution using a bent glass rod. The agar plates were incubated for 24 hr at 37 C, after which the number of colonies on each plate was determined and the number of viable cells in the original suspension was calculated.

#### Growth Curves

Forty ml broth cultures were added to 250 ml Erlenmeyer flasks with an 18 mm test tube attached to the side for optical density (O.D.) measurements (side arm flasks). The flasks were shaken on a New Brunswick Model VL platform shaker at a setting of "1" at 37 C. At 2 hr intervals the flasks were removed from the shaker and the bacterial density estimated



by measuring the transmitted light at a wave length of 535 m $\mu$  by means of a Bausch and Lomb Spectronic 20 colorimeter. Growth curves were drawn by plotting O.D. against time.

#### DNA, RNA, and Protein Determinations

DNA concentrations were determined by the Burton colorimetric test (Burton, 1956) using calf thymus DNA as a standard. RNA concentrations were measured with the orcinol method (Ashwell, 1957). Protein was determined by a modified Folin-Ciocalteu method (Lowry et al., 1951) with bovine serum albumin used as a standard.

#### Sedimentation

Sedimentation analysis was carried out on a Spinco Model E ultracentrifuge at 10 C using UV optics. The DNA solution was poured into a single sector, valve-type, synthetic boundary cell. The synthetic boundary cell was used to avoid filling the cell with a syringe. Samples were centrifuged at 14,290 RPM so that the anomalous effects previously seen with high molecular weight DNA (Rosenbloom and Schumaker, 1967) could be avoided. Photographs were taken at 16 min intervals. The films were analyzed for their O.D. with the Beckman Model RB Analytrol densitometer equipped with a film adaptor. Sedimentation coefficients were corrected to the viscosity and density of water at 20 C, and are reported in Svedberg units.

### Viscometry

Relative viscosities of DNA samples were measured with a low shear viscometer produced by Beckman Instruments Inc., Fullerton, California, the design of which was based on an instrument described by Zimm and Crothers (1962). A constant temperature of 25 C was maintained by using a temperature regulator manufactured by Bronwill Scientific Division, Will Corp., Rochester, N. Y.

### CsCl Density Gradient Centrifugation

Solid CsCl was added to DNA solutions in 1.0 M BPES, pH 7.0, to achieve a density of 1.72 g/ml. The CsCl was allowed to dissolve overnight at 4 C in order to avoid shearing the DNA by mixing. Five ml of the mixture was added to a centrifuge tube with a wide-mouthed pipette, and then the centrifuge tube was filled with mineral oil. The mixture was centrifuged in a Beckman L2-65B preparative ultracentrifuge using the SW 40 rotor at 25,000 RPM for 72 hr at 20 C. After centrifugation, the contents of each tube were collected by means of a density gradient fractionator (Pattee et al., 1968). An 18-gauge needle was used, and 2-drop fractions were collected in sterile tubes at a rate of about 1 drop/2 seconds. A total of 175-194 drops per centrifuge tube was obtained. Each fraction was diluted immediately with 1 ml 1.0 M BPES, pH 7.0, and stored at 4 C. The O.D. at 260 m $\mu$  was recorded for each fraction with a microcuvette (0.9 ml sample, 10 mm light path) and a Beckman DB Spectrometer. To determine the relative no. of transformants for the ade16, leu, and met markers, 0.1 ml of every other fraction was added to 0.9 ml of

competent cells of recipient strain 168 leu-met-ade16.

A control tube was included in each centrifugation run which contained CsCl in buffer, but no DNA. The refractive index of every tenth drop from this control tube was measured and the density was calculated from the refractive index by use of an Abbe Refractometer (Bausch and Lomb Inc., Rochester, N. Y.). The density of every fortieth drop obtained from the sample tubes was calculated also. A plot was drawn of buoyant density against drop number and this plot, together with the densities of every fortieth drop from the sample tubes, was used to estimate the location of light, hybrid, and heavy DNA peaks in the CsCl density gradient. The locations of the light (1.703 g/ml), hybrid (1.742 g/ml), and heavy (1.776 g/ml) densities were based on the values found by Szybalski et al. (1960) for the densities of light (both strands containing thymine), hybrid (one strand containing 5-bromouracil instead of thymine), and heavy (both strands containing 5-bromouracil) DNA of B. subtilis.

#### Preparation of Methylated Albumin Columns

Kieselguhr was obtained from the Johns-Mansville Products Corporation, New York City, in the grade sold as "Hyflo Supercell". Methylated albumin was purchased from Worthington Biochemical Corporation. The buffered saline solutions contained 0.025 M  $\text{Na}_2\text{HPO}_4$  and 0.025 M  $\text{NaH}_2\text{PO}_4$  plus various amounts of NaCl. The buffered saline solutions were stored in the cold.

The protein-coated kieselguhr was prepared according to Mandell and

Hershey (1960). "Washed, protein-coated kieselguhr, used for the second layer in the column, is prepared as follows. Boil (to expel air) and cool a suspension of 20 g kieselguhr in 100 ml of 0.1 M-buffered saline. Add 5 ml of 1% esterified albumin, stir, and add 20 ml of additional saline." This suspension of kieselguhr coated with methylated albumin, designated MAK, was stored at 5 C.

All columns were operated at room temperature. Two types of columns were used. Preliminary tests were performed using a column described by Sueoka and Cheng (1962). This column consists of a single layer of MAK and employs a stepwise gradient of NaCl to remove the nucleic acids. This column was formed by pouring a slurry of 1 g Whatman cellulose powder (CF 1) in 10 ml of buffered saline into a 1 cm glass tube. When the cellulose powder had settled and most of the buffered saline had passed through, 5 ml of MAK was added to the column. After the MAK had settled, the column was washed with 50 ml of buffered saline under air pressure at a flow rate of 3 ml per min. The pressure was applied directly to the column by using a rubber stopper with a glass tube connected to the air line. The concentration of the nucleic acids in the sample was adjusted to about 5-10  $\mu$ g of DNA per ml with buffered saline. It was applied to the surface of the column with as little disturbance as possible, and passed through the column under an air pressure which gave a flow rate of 2 ml per min. Elution of the nucleic acids was accomplished by passing stepwise through the column a series of buffered saline solutions of increasing salt concentrations.

The second type of MAK column, described by Mandell and Hershey

(1960), contains 3 layers. A linear gradient is employed with this column to elute the nucleic acids. This column was prepared in a 2.1 cm glass tube as follows. Suspensions of Kieselguhr in 3 beakers were boiled and cooled:

first beaker: 4 g in 20 ml of 0.1 M NaCl

second beaker: 3 g in 20 ml of 0.4 M NaCl

third beaker: 0.5 g in 5 ml of 0.4 M NaCl

One ml of 1% methylated albumin was added to the first beaker and the contents stirred, then an additional 7.5 ml of 0.1 M NaCl was added. The first layer of the column was formed on Whatman cellulose powder with this suspension. The excess protein was washed down into the column bed with 0.1 M buffered saline to avoid contaminating the material to be added next. Five ml of MAK was added to the suspension in the second beaker and immediately added to the column to form the second layer. This layer was then covered with the contents of the third beaker. The column was washed with 250 ml of 0.6 M buffered saline. The liquid displacement volume of the finished column was about 24 ml. The column was loaded with 212  $\mu$ g of DNA in 25 ml of 0.6 M buffered saline. A linear gradient of NaCl between 0.6 M and 1.0 M (each 100 ml) in the phosphate buffer was used to elute the column. No pressure was applied to this column. The % transmittance (% T) of the eluent was monitored with a Canalco UV analyzer, Canal Industrial Corporation, Rockville, Indiana, at 254 m $\mu$  and 64-drop fractions (approximately 4 ml/fraction) were collected with an LKB 3400 B Radi Rac Fraction Collector, Stockholm, Sweden.

## RESULTS

## Effect of Shearing on DNA

Nester et al. (1963) have shown that mechanical shearing of DNA from B. subtilis causes fragmentation of the DNA molecules. The DNA that had been mechanically sheared possessed a lower sedimentation value, a reduced viscosity, a decreased genetic activity, and a decreased co-transfer of linked markers.

In this experiment the effect of shearing on the genetic activity of B. subtilis DNA was followed. One ml of B. subtilis strain W23 DNA (Sample C) was added to 4 ml 1.0 M BPES. This DNA solution was pulled in and expelled (2 transfers) through a 25 gauge needle, 2 cm long, attached to a 20 cc syringe at a rate of 5 ml/30 sec. Samples of 0.1 ml were removed after 0, 2, 4, 6, and 8 transfers and assayed for their ability to transform the thr marker to recipient strain 168 leu-met-thr. The number of transformants resulting from the DNA which had been sheared through the syringe needle was divided by the number of transformants resulting from the unsheared DNA, then this value was multiplied by 100. The results are presented in Figure 2. After 2 transfers no decrease in the genetic activity developed, but after 4 transfers the relative no. of transformants dropped to 38%. No further significant decrease in activity occurred after 6 and 8 transfers. Nester et al. (1963) also found that genetic activity of DNA, after progressive shearing, rapidly decreased until a plateau level was reached. No further decrease in single marker transfers resulted from their shearing methods after the plateau level had been reached. Because there was no decrease in genetic activity after 2 transfers, all sheared DNA samples used in subsequent

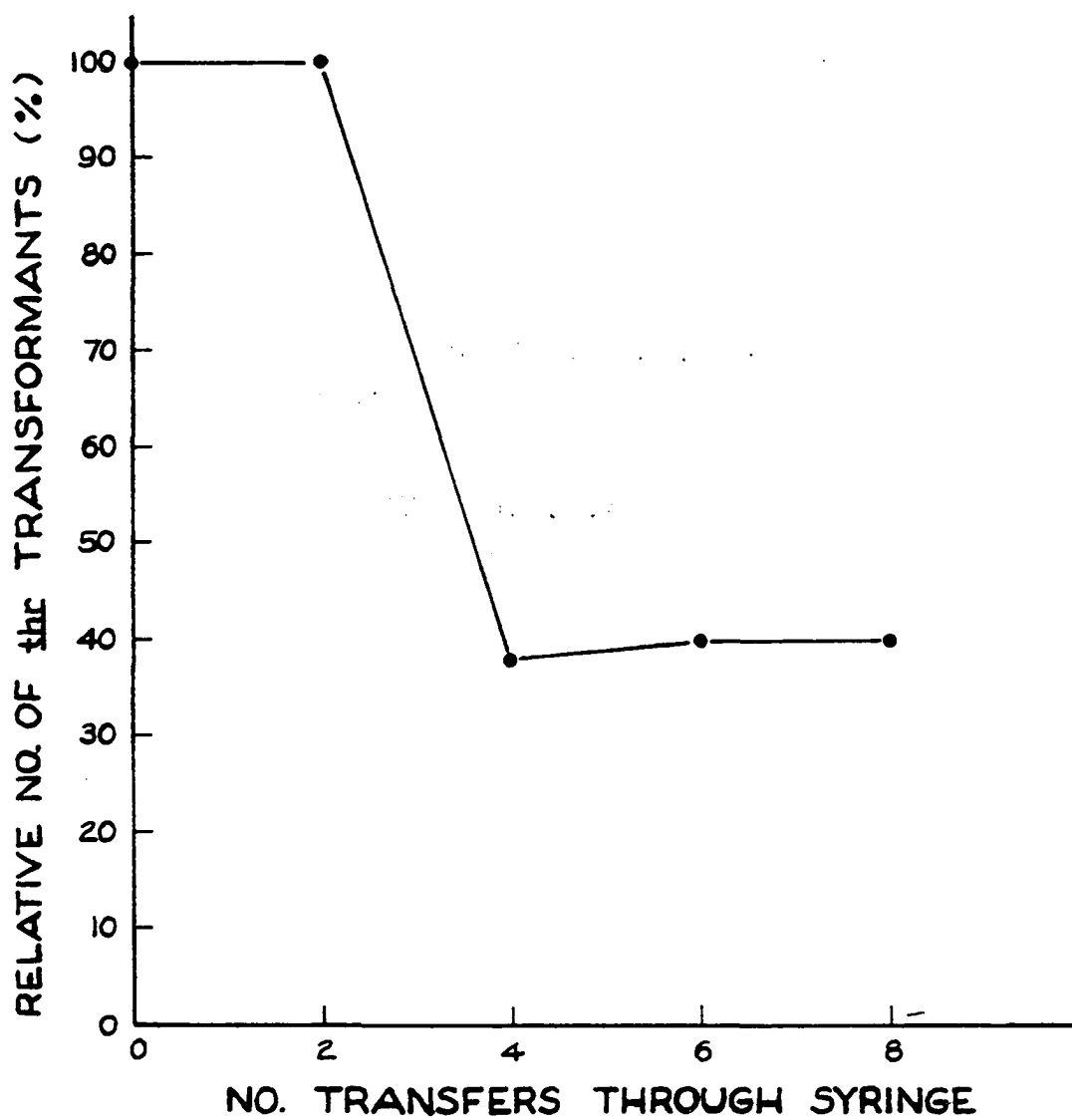


Figure 2. The effect of shearing on the genetic activity of B. subtilis DNA.

experiments were treated with 2 transfers as described here.

### Isolation of High Molecular Weight DNA

#### DNA Sample A

A 16-hr BHI agar slant culture of B. subtilis strain W23 was suspended in 10 ml of Minimal Medium and added to a Fernbach flask containing 1 L of TSB. The flask was shaken on a New Brunswick Model VL rotary platform shaker at a setting of "1". When the culture was 4 hr into the stationary phase of growth (9 hr later), the cells were centrifuged at 16,000 x g at 4 C and resuspended in 40 ml of 0.1 M BPES buffer, pH 9.0. Lysozyme was added to a final concentration of 5 mg/ml, and the suspension was poured into a dialysis bag 18 mm in diameter which had been previously treated with 64% ZnCl<sub>2</sub> for 15 min at room temperature. The dialysis bag was suspended in a 1 L graduated cylinder containing 1.0 M BPES buffer, pH 9.0. The cylinder was sitting in a water bath. After the cells had been exposed to lysozyme for 30 min at 37 C, the temperature of the water bath was raised to 50 C, and 1 mg of Pronase per ml was added to the lysate. The buffer in the graduated cylinder was changed periodically. Pronase was replenished by the addition of 0.5 mg per ml twice at 4-hr intervals. Four hr after the last Pronase treatment the pH of the 1.0 M BPES buffer was adjusted to 7.0 and dialysis continued for 24 hr. The lysate was clarified by centrifugation at 16,000 x g for 10 min and stored at 4 C. DNA Sample A contained 127 µg of protein, 108 µg of RNA, and 37.5 µg of DNA per ml. The O.D. at 260 mµ of DNA Sample A was 1.76.



DNA Samples B-1 and B-2

Half of DNA Sample A (approximately 20 ml) was treated with 0.1 mg of RNase per ml twice at 4-hr intervals, and then treated 5 times with hot phenol as described by Massie and Zimm (1965b). It was then gently poured into an 18 mm dialysis bag previously treated with  $\text{ZnCl}_2$ . The DNA sample was dialyzed at 37 C in 6 L of 1.0 M BPES, pH 7.0. After 18 hr the buffer was changed and dialysis was continued for 8 more hr. The O.D. at 260 m $\mu$  of this sample could not be measured until the sample was diluted 1:5. The 1:5 dilution gave an O.D. of 0.65 units. The sample was extracted with anhydrous ether in a 500 ml medicine bottle laid on its side. After 24 hr at 0 C,  $\text{N}_2$  was blown over the DNA to remove the ether. After the sample had been dialyzed in an 18 mm dialysis bag for 12 hr in 6 L of 1.0 M BPES, pH 7.0, the O.D. at 260 m $\mu$  was 1.40. Because the DNA, RNA, and protein present could not account for this high reading, phenol was assumed to still be present. The ether extraction was repeated for 4 days at 0 C with 4 changes of ether and  $\text{N}_2$  was blown over the DNA to remove the ether. The O.D. was still excessively high (1.2 units), perhaps because the viscosity of the DNA prevented the ether extraction from removing the rest of the phenol. The sample was then dialyzed in dialysis tubing 0.6 mm in diameter for 48 hr at 37 C in 6 L of 1.0 M BPES, pH 7.0. The buffer was changed at 24 hr. The O.D. of DNA Sample B-1 had dropped to 0.57, indicating that most of the phenol had been removed; however, an accurate determination of the protein in DNA Sample B-1 was impossible with the Folin-Ciocalteu method (Lowry et al., 1951) because traces of phenol still present interfered with the reaction. DNA Sample

B-1 contained 20.54  $\mu\text{g}$  of DNA and 24.0  $\mu\text{g}$  of RNA per ml.

DNA Sample B-2, which was isolated using the same steps employed with DNA Samples A and B-1, contained 31.68  $\mu\text{g}$  of DNA per ml.

#### DNA Sample C

A 16-hr BHI agar slant culture of B. subtilis strain W23 was suspended in 10 ml of Minimal Medium and added to a Fernbach flask containing 1 L of Standard Medium. The flask was shaken on a New Brunswick Model VL rotary platform shaker at a setting of "1". When the culture was 4 hr into the stationary phase of growth, 0.3% PEA was added. The cells were incubated for 2 hr at 30 C without shaking. The cells were collected by centrifugation at 16,000  $\times g$  at 4 C using a Servall RC-2B centrifuge with a GSA head and resuspended in 40 ml of 1.0 M BPES, pH 9.0. A sample removed at this time showed 262  $\mu\text{g}$  of protein, 159.5  $\mu\text{g}$  of RNA, and 46.6  $\mu\text{g}$  of DNA per ml. Five mg of lysozyme per ml and 1 mg of RNase per ml were added and the solution poured into a  $\text{ZnCl}_2$ -treated dialysis bag 18 mm in diameter. The dialysis bag was suspended in a 1 L graduated cylinder containing 1.0 M BPES, pH 9.0 and sitting in a 37 C water bath. After  $\frac{1}{2}$  hr the temperature was raised to 50 C and 2 mg of Pronase per ml were added. A sample removed 12 hr later showed 750  $\mu\text{g}$  of protein, 82.6  $\mu\text{g}$  of RNA, and 46.8  $\mu\text{g}$  of DNA per ml. An additional 0.5 mg of Pronase per ml was added and dialysis continued for 24 hr. A sample removed at this time showed 243  $\mu\text{g}$  of protein, 49.3  $\mu\text{g}$  of RNA, and 42.2  $\mu\text{g}$  of DNA per ml. Dialysis was continued for 12 hr more; a sample was then removed

which showed 132  $\mu\text{g}$  of protein, 42.8  $\mu\text{g}$  of RNA, and 42.2  $\mu\text{g}$  of DNA per ml. After 28 hr of dialysis a sample was removed which revealed 83.4  $\mu\text{g}$  of protein, 46.4  $\mu\text{g}$  of RNA, and 44.2  $\mu\text{g}$  of DNA per ml. The temperature of the water bath was lowered to 37 C and, when the temperature of the buffer was 37 C, 1 mg of RNase per ml was added. After 3 hr, the temperature was raised to 50 C and 0.5 mg of Pronase per ml added. Twenty-four hr later a sample was taken which revealed 150.2  $\mu\text{g}$  of protein, 47.6  $\mu\text{g}$  of RNA, and 43.4  $\mu\text{g}$  of DNA per ml. With a wide-mouthed pipette, the lysate was transferred to  $\text{ZnCl}_2$ -treated dialysis tubing 6 mm in diameter. Tubing of this size had proved to be very effective in removing phenol by dialysis. Dialysis was continued for 24 hr at 50 C, at which time DNA Sample C was poured into a sterile 125 ml Erlenmeyer flask and stored at 4 C. DNA Sample C contained 67.8  $\mu\text{g}$  of protein, 33.0  $\mu\text{g}$  of RNA, and 42.4  $\mu\text{g}$  of DNA per ml. Ninety-one % of the DNA was recovered, while 73% of the protein and 79% of the RNA were removed. When several 0.1-ml quantities were spread by means of a glass rod on the surface of a TSA plate, no colonies were formed after incubation at 37 C for 24 hr, indicating sterility.

#### Molecular Weight Estimations

##### Viscometry

The intrinsic viscosities of DNA Samples A, B-1, and B-2 were measured using a low shear viscometer (Zimm and Crothers, 1962). Dilutions of DNA were gently mixed and stored for 24 hr at 4 C prior to use. The stator and rotor of the viscometer were rinsed between each measure-

ment and filled with fresh sample. Table 5 contains the viscosity data for DNA Sample A. The data were treated as described by Crothers and Zimm (1965). The least squares method was used to find the line of best fit when  $\ln \eta_{rel}/c$  was plotted against concentration. The intercept of this line, at zero concentration, is the intrinsic viscosity,  $[\eta]$ . The value for the intrinsic viscosity of DNA Sample A is 489.33 dl/gm. This value is very close to the intrinsic viscosity of 500 dl/gm reported by Massie and Zimm (1965a) for B. subtilis DNA. The molecular weight was calculated to be  $3.05 \times 10^8$  daltons using the equation  $0.665 \log_{10} M = 2.863 + \log_{10} [\eta] + 5$  (Crothers and Zimm, 1965) and the intrinsic viscosity of 489.33 dl/gm.

Table 5. Viscosity of B. subtilis strain W23 DNA Sample A, which had been treated by Pronase alone

Concentration (g/dl $\times 10^4$ )	sec/rev <sup>a</sup>	$\eta_{rel}$	$\ln \eta_{rel}/c$ (g/dl)
0	136	1.0	
(1.0 M BPES)	138		
37.5	634 550	4.32	390.2
25.0	383 369	2.74	403.2
12.5	228 261	1.78	461.3

<sup>a</sup>Rotor and stator rinsed between each measurement and stator filled with fresh sample.

When Massie and Zimm (1965a) treated their DNA samples with hot phenol after Pronase, the viscosity did not appear to change. This was not true for DNA Samples B-1 and B-2. The viscosity data for DNA Samples B-1 and B-2 are presented in Table 6. From the plot of  $\ln \eta_{rel}/c$

Table 6. Viscosity of B. subtilis strain W23 DNA Samples B-1 and B-2 which were treated with Pronase and hot phenol

Sample	Concentration (g/dl x 10 <sup>4</sup> )	sec/3 rev <sup>a</sup>	$\eta_{rel}$	$\ln \eta_{rel}/c(\text{g/dl})$
1.0 M BPES	0	376 380 390 384 375 378	1.0	
B-1	20.54	631 604	1.65	243.8
B-1	10.27	477 486	1.27	231.0
B-1	6.85	440 452	1.17	234.3
B-1	4.11	415 429	1.11	240.9
B-2	31.68	930 916	2.45	283.5
B-2	21.12	692 687	1.84	287.5
B-2	15.84	578 578	1.54	272.6
B-2	10.56	521 515	1.38	305.0

<sup>a</sup>Rotor and stator rinsed between each measurement and stator filled with fresh sample.

against concentration, the least squares method was used to extrapolate to an intrinsic viscosity of 281.4 dl/g. This corresponded to a molecular weight of  $9.99 \times 10^7$  daltons for the phenol-treated DNA Samples B-1 and B-2.

### Sedimentation

Originally it had been planned to use DNA samples treated with hot phenol in sedimentation velocity measurements but when it became so difficult to remove the phenol, DNA Sample A was used instead. Sedimentation coefficient values were obtained at three different concentrations and are presented in Table 7. Crothers and Zimm (1965) suggest plotting the reciprocal of the sedimentation coefficient against concentration. The intercept of this line at zero concentration is the reciprocal of the sedimentation coefficient,  $S_{w,20}^0$ . The  $S_{w,20}^0$  was found to be 79.16 Svedberg units for DNA Sample A. This is in good agreement with the value of 81 obtained by Massie and Zimm (1965a). The molecular weight was calculated to be  $2.09 \times 10^8$  daltons from the equation  $0.445 \log_{10} M = 1.819 + \log_{10}(S_{w,20}^0 - 2.7)$  (Crothers and Zimm, 1965).

Table 7. The sedimentation coefficient values for 3 different concentrations of DNA Sample A

Concentration $\mu\text{g/ml}$	$S_{w,20}$	$1/S_{w,20}$
37.5	37.566	0.02660
25.0	47.500	0.02103
18.75	50.121	0.01995

## Methylated Albumin Columns

Stepwise elution from methylated albumin columns

Studies performed using the MAK column of Sueoka and Cheng (1962) indicated that the initial NaCl concentration used for application of the sample must be below 0.70 M, even though DNA of B. subtilis was not eluted until the molarity was raised to 0.80. In Table 8 the starting concentration of NaCl was 0.64. The sample applied to the column contained 96  $\mu$ g of strain W23 DNA (Sample C) in 30 ml of 0.64 M buffered saline. After the sample had been washed through with 20 ml of 0.64 M buffered saline, then 10 ml quantities of buffered saline at 0.66 M, 0.68 M, 0.70 M, 0.75 M, 0.80 M, 0.85 M, and 0.90 M were washed through the column.

Fractions of 5 ml were collected and assayed for transforming activity using recipient strain 168 leu-met-thr. As indicated in Table 8, the DNA was eluted only after the molarity had been raised to 0.80. Based on O.D. at 260 m $\mu$ , only 19% of the DNA applied to the column was recovered. When the initial salt concentration used to equilibrate the column was raised to 0.70 M, the sample passed directly through the column. This experiment was repeated 2 times and each time the DNA passed directly through the column when the initial salt concentration was 0.70 M. Not until the salt concentration was dropped to 0.64 M did the DNA absorb.

Table 8. Stepwise elution of B. subtilis strain W23 DNA from a MAK column

Fraction number	Volume collected (ml)	Eluting buffer (M of NaCl)	O.D. <sub>260</sub>	No. transformants/0.1 ml transformation mixture <sup>a</sup>		
				<u>thr</u>	<u>met</u>	<u>leu</u>
1	5	0.64	0.015	0	0	0
2	15	0.64	0.015	0	0	0
3	15	0.64	0.025	0	0	0
4	20	0.64	0.015	0	0	0
5	5	0.66	0.010	0	0	0
6	5	0.66	0.040	0	0	0
7	5	0.68	0.012	0	0	0
8	5	0.68	0.015	0	0	0
9	5	0.70	0.010	0	0	0
10	5	0.70	0.010	0	0	0
11	5	0.75	0.007	0	0	0
12	5	0.75	0.017	0	0	0
13	5	0.80	0.012	240	205	310
14	5	0.80	0.032	3075	2195	2710
15	5	0.85	0.025	1790	1180	1535
16	5	0.85	0.020	890	780	1015
17	5	0.90	0.010	600	565	750
18	5	0.90	0.015	780	520	820

<sup>a</sup>Each number is the mean of duplicate plate counts.



Gradient elution of unsheared DNA from a methylated albumin column

Five ml of B. subtilis strain W23 DNA (Sample C) contained in 1.0 M BPES were gently mixed with 4.0 ml 0.1 M buffered saline and 16 ml 0.6 M buffered saline. This sample, containing 212 µg of DNA in 25 ml of 0.6 M (final molarity) buffered saline, was applied to a Mandell and Hershey (1960) MAK column; the column was washed with 75 ml of 0.6 M buffered saline. A linear gradient of NaCl between 0.6 M and 1.0 M was applied to the column and 64-drop fractions were collected. Each fraction was assayed for its ability to transform recipient strains 168 leu-met-ade16 and 168 leu-met-thr. The ade and leu marker frequencies were tested with strain 168 leu-met-ade16, while the thr and met marker frequencies were tested with strain 168 leu-met-thr. The results of this experiment, presented in Figure 3, do not demonstrate any enrichment of markers. To examine the possibility that saturating levels of DNA in the fractions might be interfering with the demonstration of marker enrichment, each fraction was diluted 1:10 into 1.0 M BPES and reexamined for the ability to transform recipient strains 168 leu-met-ade16 and 168 leu-met-thr. As can be seen in Figure 4, there is no apparent marker enrichment with the 1:10 dilutions of the fractions.

The DNA began to elute from the column with 0.66 M NaCl and continued to elute until the molarity reached 0.77 M. Saito and Masamune (1964) also found that B. subtilis DNA is eluted with 0.66 M NaCl from this type of column. When they used a Mandell and Hershey (1960) MAK column the DNA eluted in a rather broad peak similar to that shown in Figure 3. But, contrary to the results of Saito and Masamune (1964), no marker

Figure 3. The gradient elution of unsheared *B. subtilis* strain W23 DNA from a Mandell and Hershey (1960) MAK column. The ade (●) and leu (■) marker frequencies were examined with strain 168 leu-met-ade16, while the thr (○) and met (▲) marker frequencies were tested with strain 168 leu-met-thr.

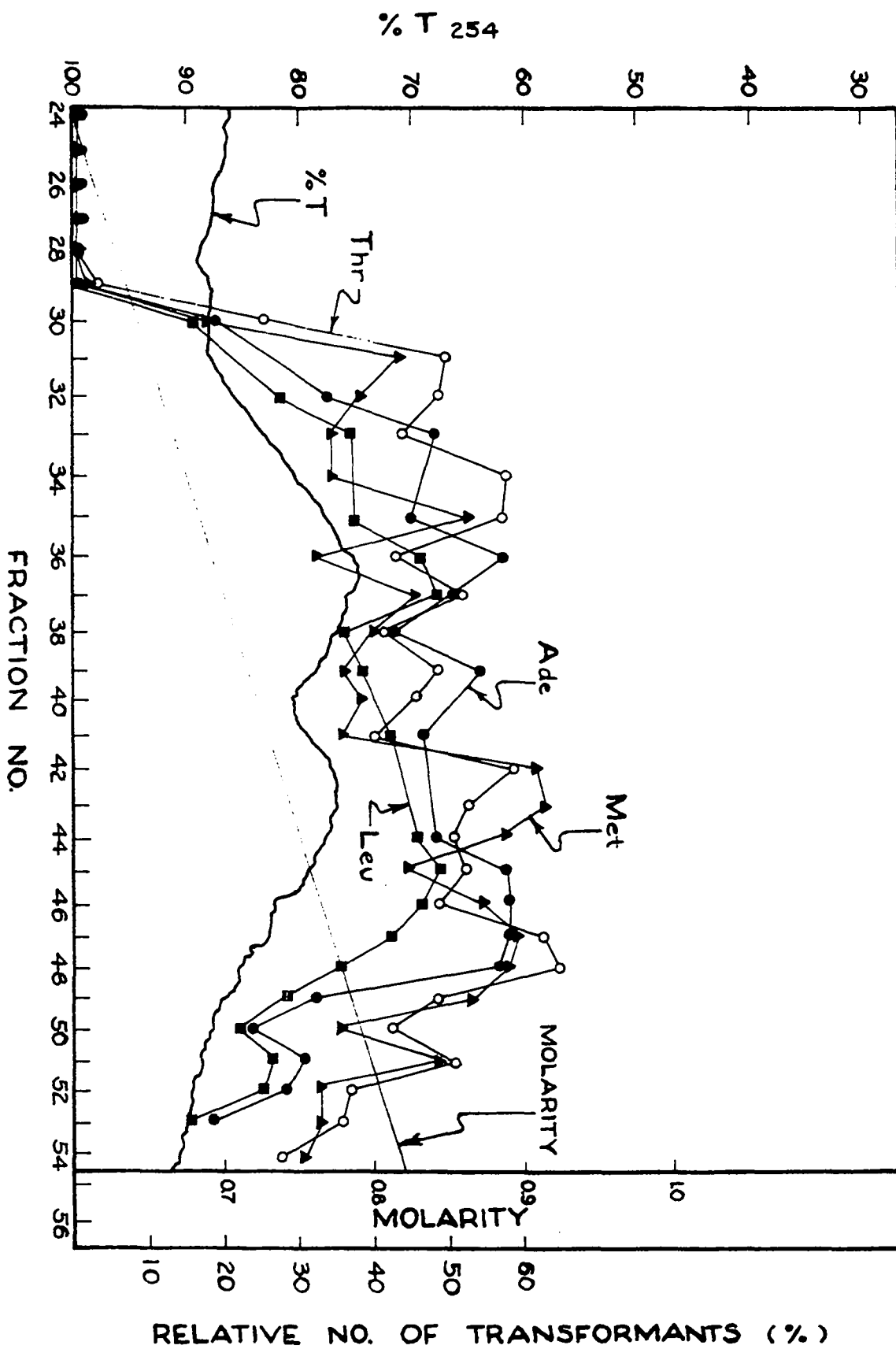
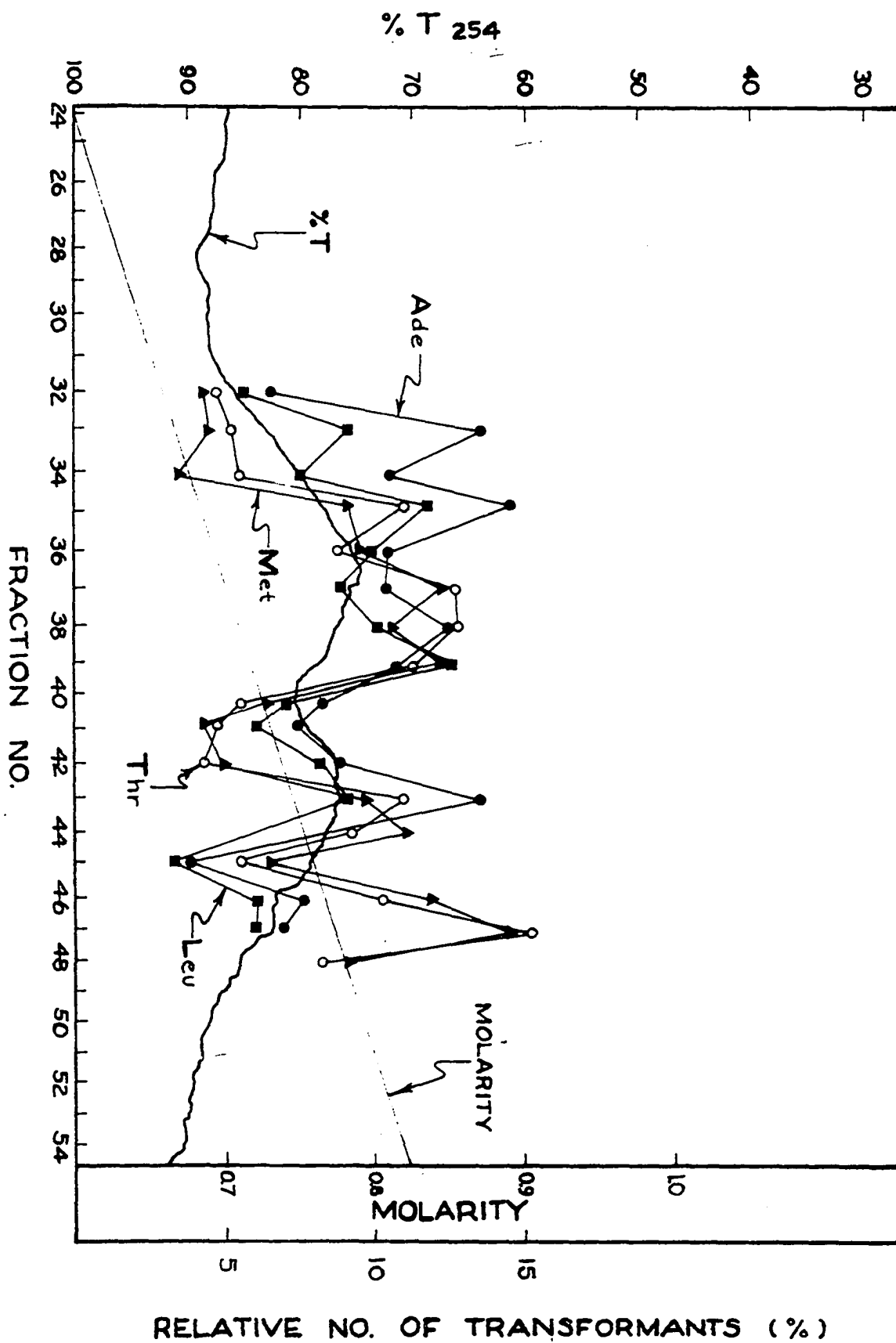


Figure 4. The gradient elution of unsheared B. subtilis strain W23 DNA from a Mandell and Hershey (1960) MAK column. The ade (●) and leu (■) marker frequencies were examined with strain 168 leu-met-ade16, while the thr (○) and met (▲) marker frequencies were tested with strain 168 leu-met-thr.



enrichment could be demonstrated in the experiments performed with the unsheared DNA (Figures 3 and 4).

Gradient elution of sheared DNA from a methylated albumin column

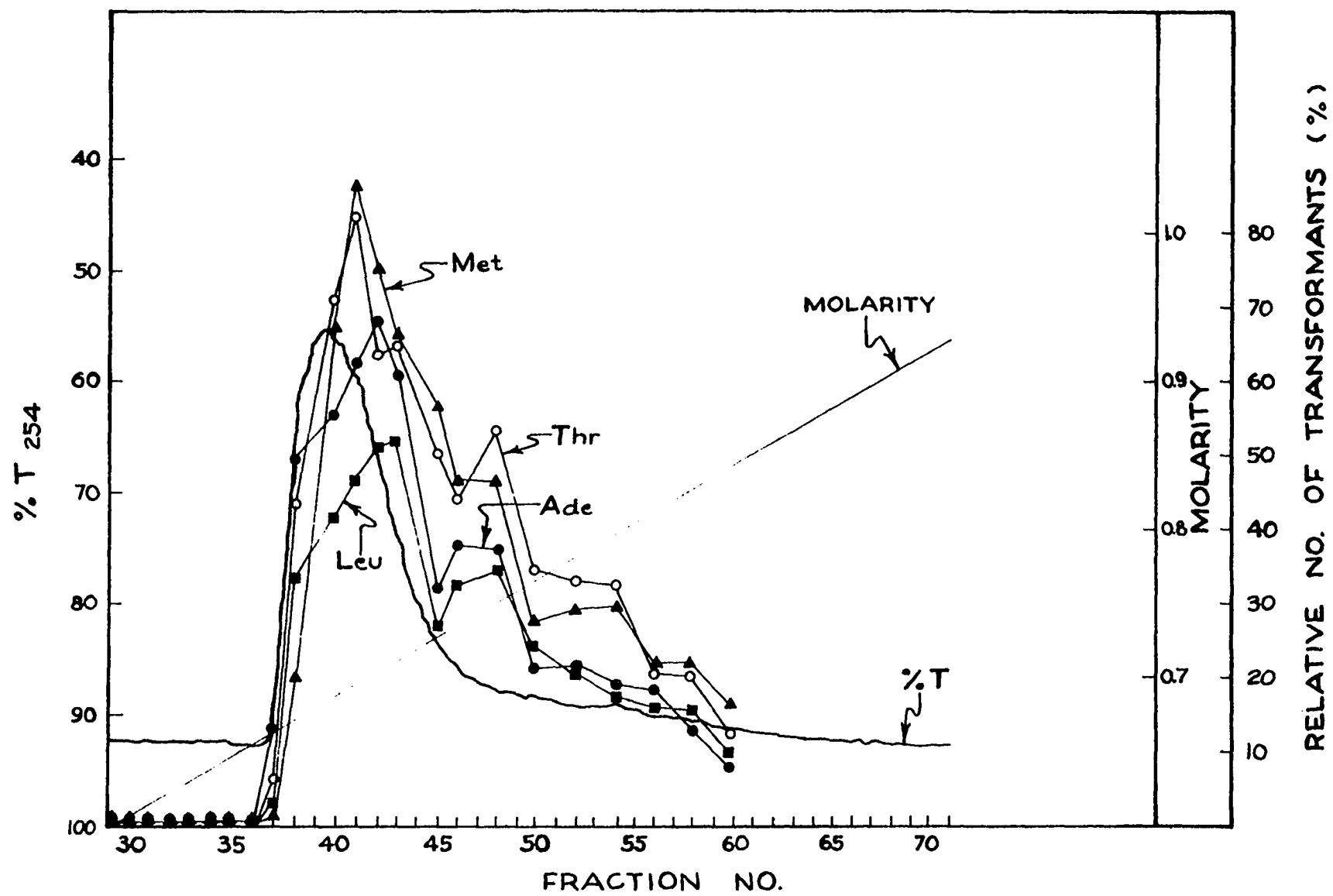
A sheared DNA sample was examined to determine whether the MAK column would fractionate the fragmented DNA molecules into different genetic sizes. A DNA sample (212  $\mu$ g of DNA Sample C in 25 ml of 0.6 M buffered saline) was prepared in the same manner as the DNA sample used in the gradient elution of unsheared DNA from a MAK column. The sample was then sheared by passage through a syringe needle as previously described. In contrast to the previous results (Figure 2), there was a decrease in the genetic activity for the DNA sample after 2 transfers through the syringe (Table 9). All of the markers seemed equally sensi-

Table 9. The effect of shearing on the ability of DNA to transform strain 168 leu-met-ade16

Sample	Relative no. of transformants (%)		
	ade	leu	met
Unsheared DNA	100.0	100.0	100.0
Sheared DNA (2 transfers)	33.0	33.4	34.5

tive to the effects of shearing. The sheared sample was applied to a Mandell and Hershey (1960) MAK column and the column was treated in the same manner as described for the gradient elution of unsheared DNA from a MAK column. The gradient elution pattern of the sheared DNA sample (Figure 5) was very different from the gradient elution pattern of the

Figure 5. The gradient elution of sheared B. subtilis strain W23 DNA from a Mandell and Hershey (1960) MAK column. The ade (●) and leu (■) marker frequencies were examined with strain 168 leu-met-ade16, while the thr (○) and met (▲) marker frequencies were tested with strain 168 leu-met-thr.





unsheared DNA sample (Figure 3). Instead of eluting in broad peaks as did the unsheared DNA in Figure 3, the sheared DNA sample eluted in one sharp peak (Figure 5). As in the case of the unsheared DNA, the sheared DNA began to elute from the column when the molarity of NaCl was raised to 0.66. All fractions in the peak appeared equally able to transform all 4 markers, indicating that there was no marker enrichment in any of the fractions.

### Phenethyl Alcohol Studies

The ultimate aim of the phenethyl alcohol (PEA) studies was to determine the most appropriate concentration of PEA to allow completion of a replicating chromosome but not allow reinitiation of replication. Treick and Konetzka (1964) used concentrations of PEA between 0.28 and 0.32% in their studies of E. coli. They found that exponentially growing cultures of E. coli in a semisynthetic medium, when inhibited with concentrations of PEA between 0.28 and 0.32%, continued to synthesize DNA for 1 hr, after which DNA synthesis stopped. The increases in DNA were always 40 to 60%. At these concentrations, cell division was inhibited for at least 4 hr with no significant loss of viability.

The effect of different PEA concentrations on the viability of B. subtilis was determined. Strain W23 was grown on a BHI agar slant for 12 hr at 37 C. The slant culture was suspended in 10 ml of Minimal Medium, and this cell suspension was added to 1 L of Medium S contained in a Fernbach flask. After shaking for 4 hr at 37 C on a New Brunswick

Platform shaker at a setting of "1", 50-ml quantities of the cell suspension were added to 5 250-ml side arm flasks. At this time 0.375, 0.400, 0.425, and 0.450% PEA was added separately to 4 of the flasks and the flasks shaken for an additional 4 hr. During this experiment, the O.D. at 535 m $\mu$  was recorded and viable cell counts performed at 2 hr intervals. As can be seen from the summarization of the results in Figure 6, all concentrations of PEA from 0.375 to 0.450% adversely affected viability. Even though an increase in turbidity was noted with 0.375% PEA, there was a concomitant decrease in cell number. An unexpected result of this experiment was that 0.450% PEA appears to have lysed the cells, as evidenced by a 5-fold decrease in turbidity.

The experiment was repeated to determine the effect on cell viability of PEA concentrations below 0.375%. PEA, at 0.300, 0.325, and 0.350%, was added after 3 hr of growth. Viable cell counts were performed at 1 and 2 hr. The results (Table 10) reveal that all 3 concentrations allowed an initial cell increase which was inhibited after 1 hr of exposure to PEA. The concentration of PEA used in the 5-bromodeoxyuridine (5-BUDR) labeling experiments was 0.300%. As can be seen in Table 12, DNA synthesis did continue in the presence of 0.300% PEA, a result which is in agreement with Treick and Konetzka (1964).

#### Cell Lysis by Phenethyl Alcohol

The lytic effect seen with concentrations of PEA above 0.400% (Figure 6) was reexamined with E. coli B, S. aureus strain 655,

Figure 6. The effect of different concentrations of PEA on turbidity (A) and viability (B) of an actively growing culture of B. subtilis strain W23.

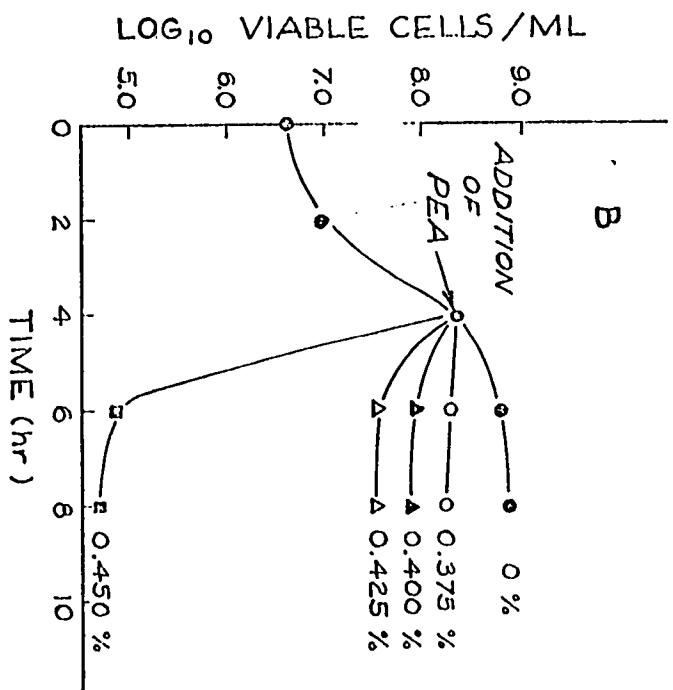
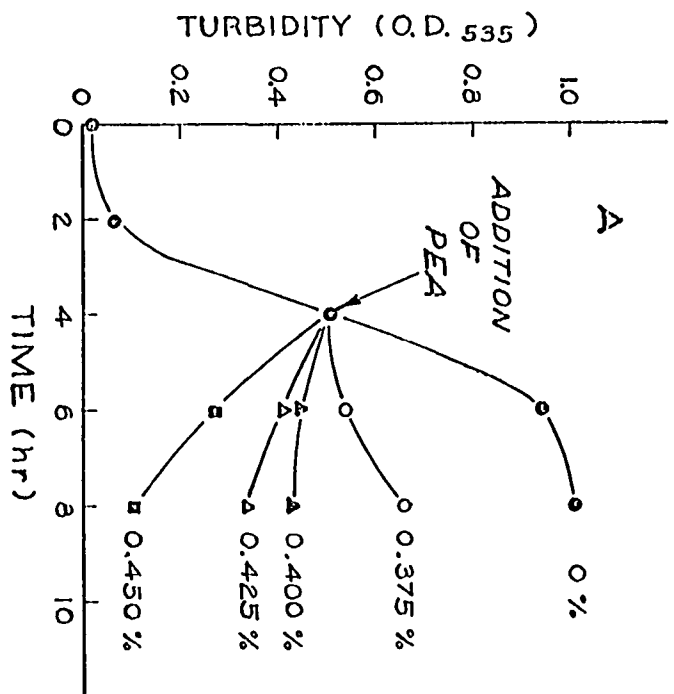


Table 10. The effect of PEA on the viable cell count of actively growing cells of B. subtilis strain W23

% PEA	Viable cells/ml		
	0 hr	1 hr	2 hr
0	$5.50 \times 10^7$	$2.04 \times 10^8$	$3.41 \times 10^8$
0.300	$5.50 \times 10^7$	$1.03 \times 10^8$	$1.15 \times 10^8$
0.325	$5.50 \times 10^7$	$1.00 \times 10^8$	$1.16 \times 10^8$
0.350	$5.50 \times 10^7$	$8.70 \times 10^7$	$8.63 \times 10^7$

and B. subtilis strain W23 cultures in both the exponential and stationary phase of growth. Twelve hr BHI agar slant cultures of the 3 organisms were suspended in 10 ml of Minimal Medium. The cell suspension of B. subtilis strain W23 was added to 1 L of Medium S in a Fernbach flask, while the cell suspension of E. coli B was added to 1 L of E medium and the cell suspension of S. aureus strain 655 was added to 1 L of Staphylococcus synthetic medium. The flasks were shaken at 37 C with a New Brunswick Rotary Platform shaker at a setting of "1". When the cells were growing exponentially, as determined from O.D. measurements at 535 mμ, 50-ml quantities were transferred into side arm flasks and 0, 0.350, 0.400, 0.450, and 0.500% PEA was added. These flasks were shaken at 37 C and the O.D. recorded periodically at 535 mμ.

To test the effect of PEA on stationary phase cells, 0.5 and 1.0% PEA was added to cultures of B. subtilis strain W23, S. aureus strain 655, and E. coli B which had been shaken for 24 hr. Shaking was con-

tinued and the O.D. was recorded periodically at 535 m $\mu$  for 24 hr. A control culture containing no PEA was included for each organism.

The results are summarized in Table 11.

Figure 7 demonstrates that exponentially growing cells of B. subtilis strain W23 are lysed completely after shaking for 3 hr in the presence of 0.450 or 0.500% PEA. When 1% PEA was added to cells from the stationary phase of growth (Table 11) the O.D. at 535 m $\mu$  dropped from 1.05 to 0.71 in 24 hr, indicating that cells in the stationary phase of growth are much less sensitive to PEA than cells which are rapidly dividing.

Table 11. Effect of PEA on turbidity of stationary phase cultures of B. subtilis strain W23, S. aureus strain 655, and E. coli B

Time <sup>a</sup> (hr)	O.D. at 535 m $\mu$								
	<u>S. aureus</u> strain 655			<u>B. subtilis</u> strain W23			<u>E. coli</u> B		
	0.0%	0.5%	1.0%	0.0%	0.5%	1.0%	0.0%	0.5%	1.0%
	PEA	PEA	PEA	PEA	PEA	PEA	PEA	PEA	PEA
24	1.50	1.50	1.50	1.05	1.05	1.05	1.22	1.22	1.22
27	1.70	1.70	1.70	1.05	1.00	0.95	1.30	1.30	1.30
28	1.90	1.90	1.90	1.05	0.99	0.94	1.50	1.40	1.50
29	1.90	1.90	1.90	1.05	0.98	0.90	1.40	1.40	1.40
30	1.90	1.90	1.90	1.05	0.96	0.87	1.40	1.40	1.40
48	1.90	1.90	1.90	1.02	0.90	0.71	1.40	1.50	1.40

<sup>a</sup>Time refers to total age of culture. PEA was added at 24 hr.

When 0.350 to 0.500% PEA was added to an exponentially growing culture of E. coli B, a turbidity decrease was detectable at all concentrations (Figure 8), although there was only a 50% reduction in turbidity after 4 hr of shaking with the highest concentration employed, 0.500%. The experiment was repeated using 0.5, 0.6, 0.8, and 1.0% PEA to determine the effect of higher concentrations on actively growing cells of E. coli B. As shown in Figure 9, after 1 hr in the presence of 1.0% PEA there was almost a 50% reduction in turbidity. Very little reduction in turbidity took place between 1 and 2 hr with 1.0% PEA. When 0.5 and 1.0% PEA was added to E. coli B cultures from the stationary phase of growth, no lysis was detectable (Table 11).

Although stationary cultures of S. aureus strain 655 were not affected by PEA (Table 11), exponentially growing cultures were sensitive to PEA but not at concentrations of PEA between 0.350 and 0.500% (Figure 10). When the experiment was repeated using concentrations of PEA between 0.5 and 1.0%, the turbidity increased in all cultures containing 0.8% or less PEA (Figure 11). The experiment was again repeated using S. aureus strain 655 cells from the early exponential phase and concentrations of PEA between 0.8 and 2.0%. In Figure 12 1.0 and 1.2% PEA have reduced the turbidity 50%. An interesting aspect of Figure 12 is that, when 2.0% PEA was used, the turbidity of the culture remained constant, indicating that no cell increase and no lysis had occurred. If, in order for lysis to occur with PEA, bacterial cells must be rapidly dividing, then perhaps 2.0% PEA caused instant death of S. aureus so that the lytic action of PEA did not have time to be effective.

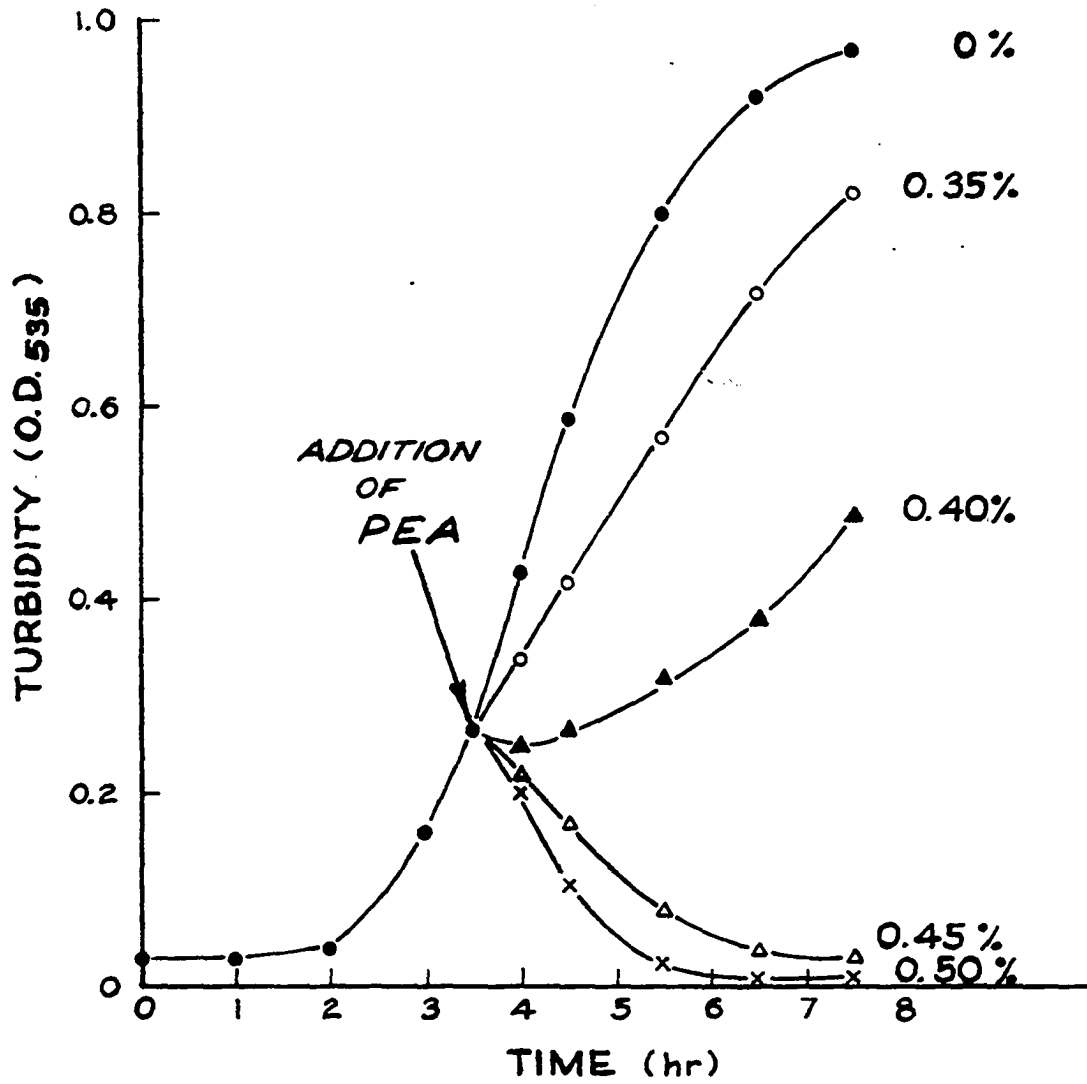


Figure 7. The effect of PEA on the turbidity of an exponentially growing culture of *B. subtilis* strain W23.



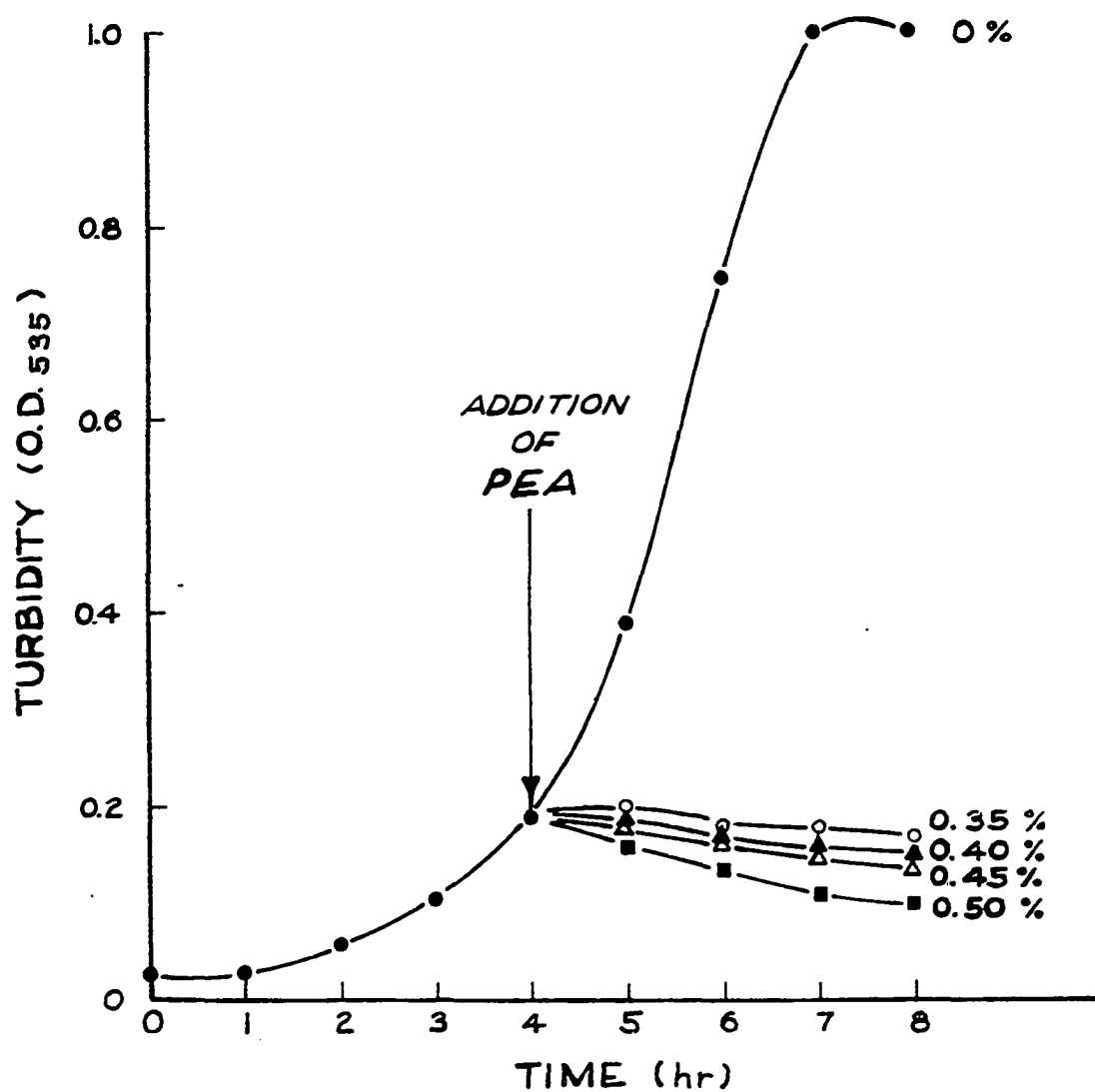


Figure 8. The effect of PEA on the turbidity of an exponentially growing culture of *E. coli* B.

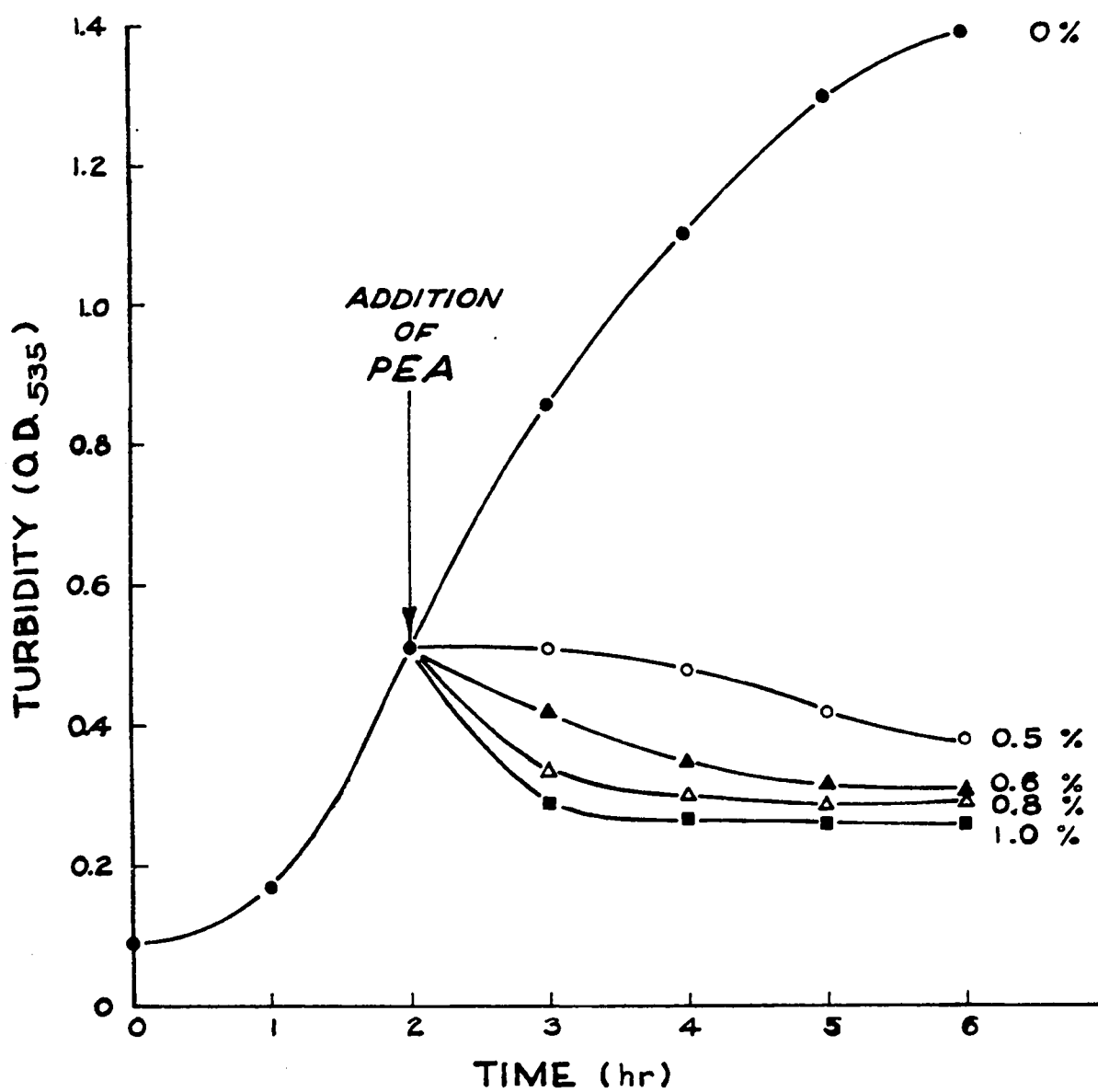


Figure 9. The effect of PEA on the turbidity of an exponentially growing culture of *E. coli* B.

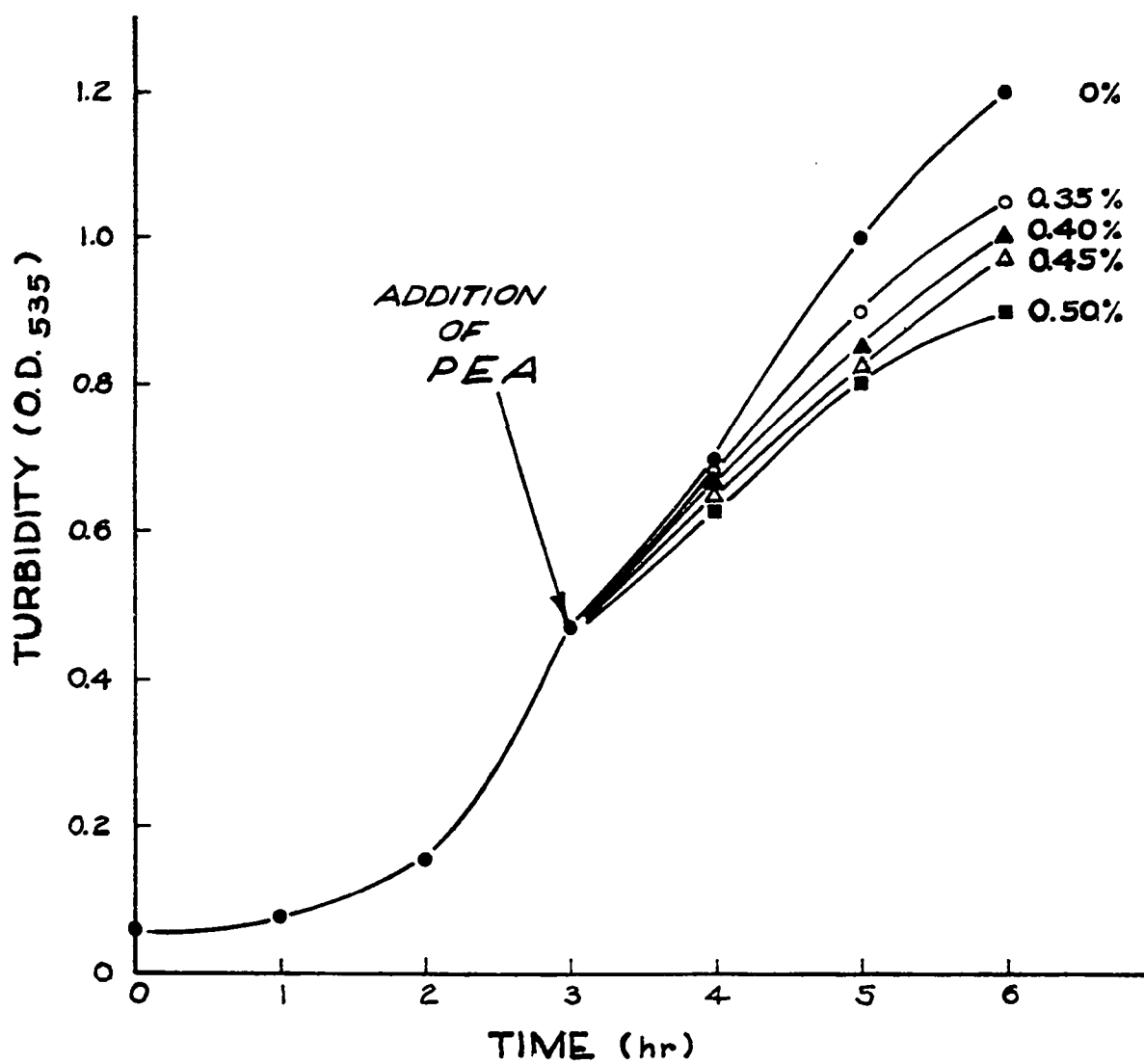


Figure 10. The effect of PEA on the turbidity of an exponentially growing culture of *S. aureus* strain 655.

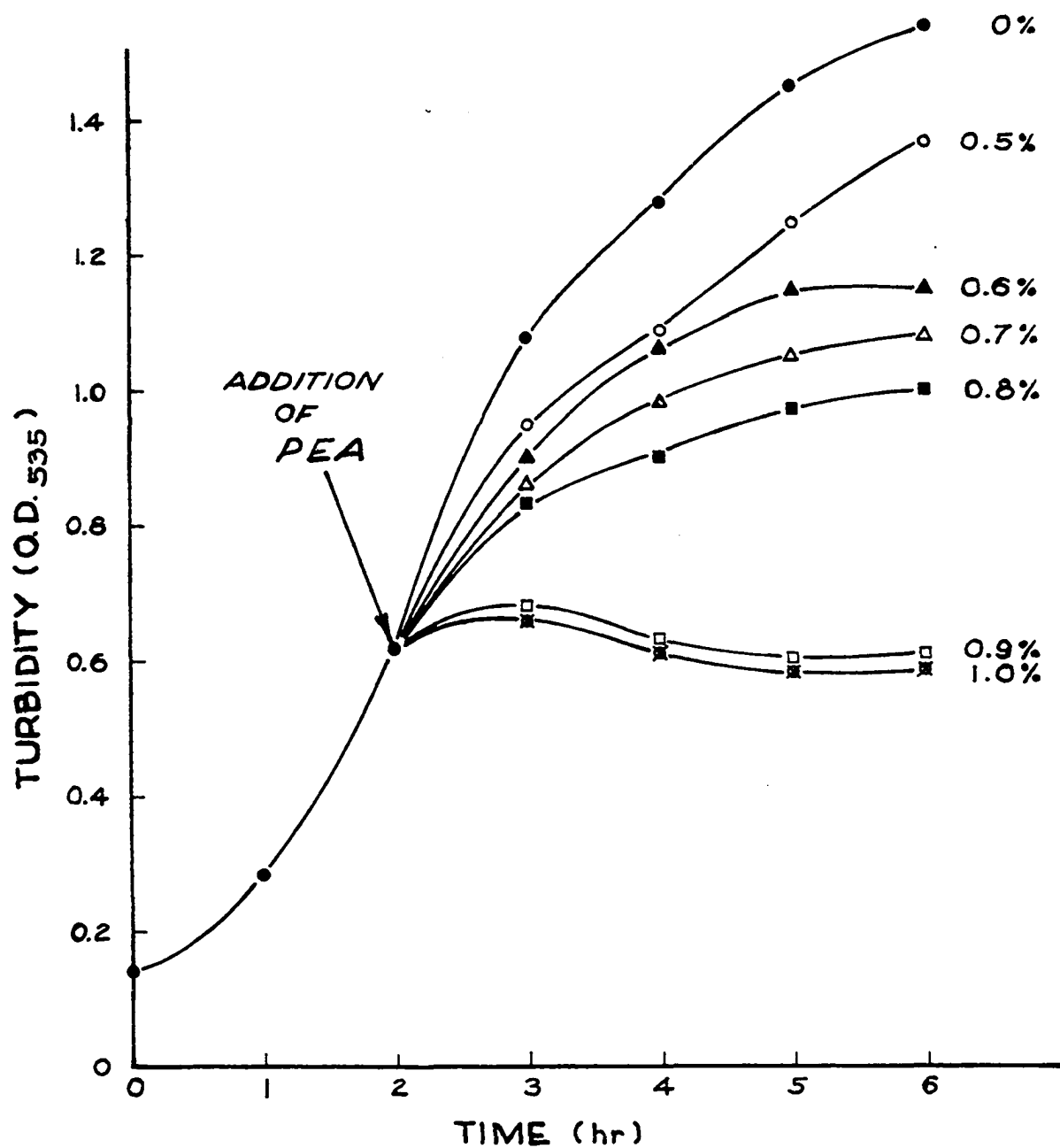


Figure 11. The effect of PEA on the turbidity of an exponentially growing culture of *S. aureus* strain 655.

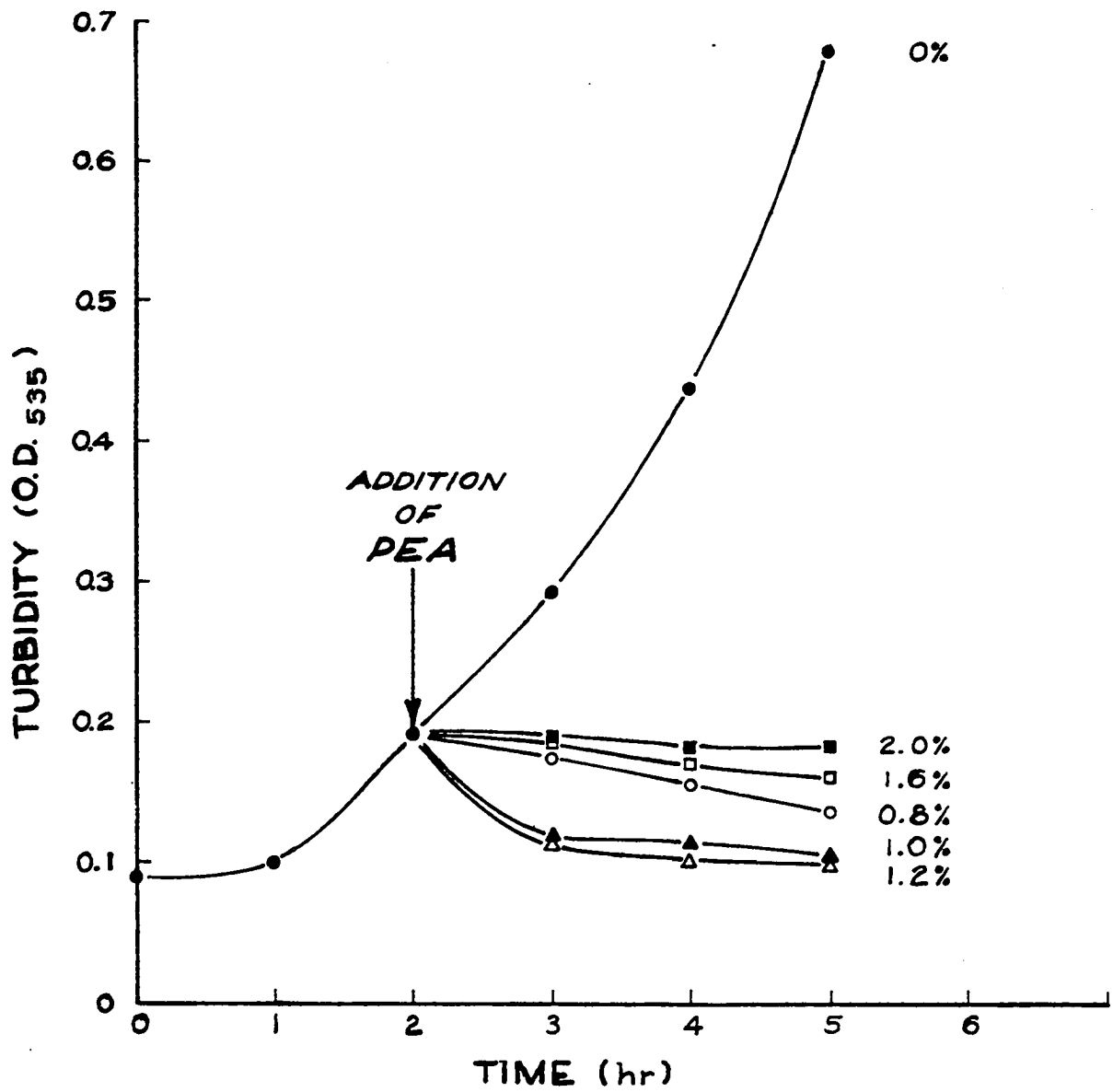


Figure 12. The effect of PEA on the turbidity of an exponentially growing culture of *S. aureus* strain 655.

Labeling the B. subtilis Chromosome with  
5-Bromodeoxyuridine

Spore germination studies

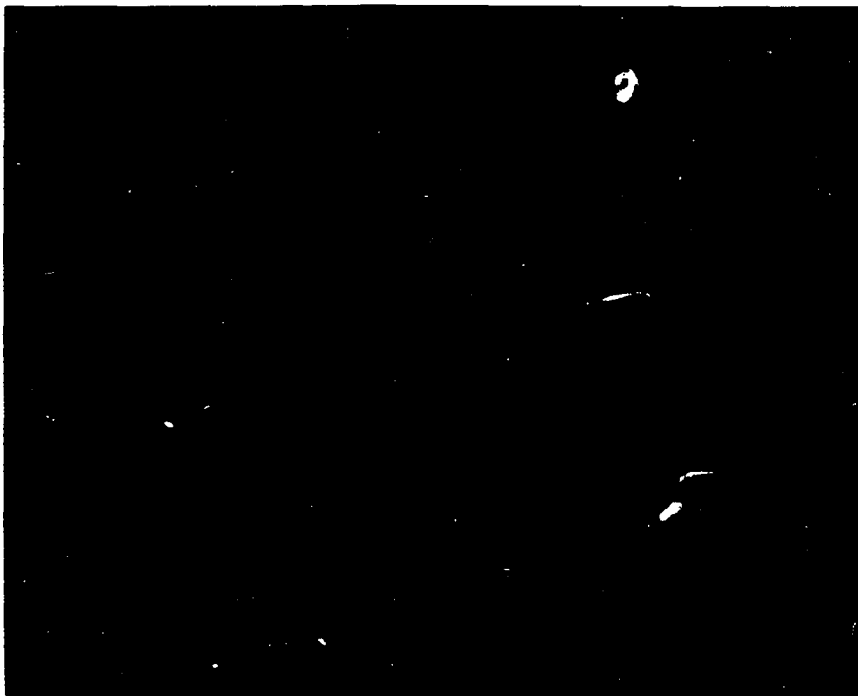
The spore preparation of B. subtilis strain 23 thy-his that was used in the 5-BUDR labeling experiments contained  $5.1 \times 10^{10}$  spores per ml. Germination studies were performed to determine the time of the first and second division in the presence of 5-BUDR and TdR. A 1:100 dilution of the spores was made into 2 side arm flasks containing 40 ml of Germination medium supplemented with 100  $\mu$ g of histidine per ml and 25  $\mu$ g of either TdR or 5-BUDR per ml. Growth was followed turbidimetrically at 37 C and the germinating spores were examined at 15 min intervals for the presence of septa. Figures 13A and 13B are photographs of a cell with one septum and a cell with 3 septa demonstrating the staining technique of Neville and Holt (1968). After 1 hr of shaking, approximately 75% of the spores in both flasks had germinated. In the flask containing TdR, 90% of the cells showed a septum after  $2\frac{1}{2}$  hr, and after  $3\frac{1}{2}$  hr approximately 50% of the cells had 3 septa. This observation indicates that the first division occurred at  $2\frac{1}{2}$  hr, while the second division occurred after  $3\frac{1}{2}$  hr in the TdR culture. Although there was little difference in growth as measured by turbidity between the TdR culture and the 5-BUDR culture (Figure 14), at  $2\frac{1}{2}$  hr only 10% of the cells from the 5-BUDR culture contained a septum. After  $3\frac{1}{2}$  hr, even though the cells from the 5-BUDR culture had increased from 1.5  $\mu$  to 6 or 8  $\mu$  in length, only 10% of the cells contained a septum. Therefore, no division time for the 5-BUDR culture could be estimated.

Figure 13. Positive phase contrast photomicrographs of B. subtilis strain W23 stained by the method of Neville and Holt (1968). X2000

- (A) a cell of strain W23 showing a single septum  
(first division)
- (B) a cell of strain W23 showing 3 septa  
(second division)



A



B



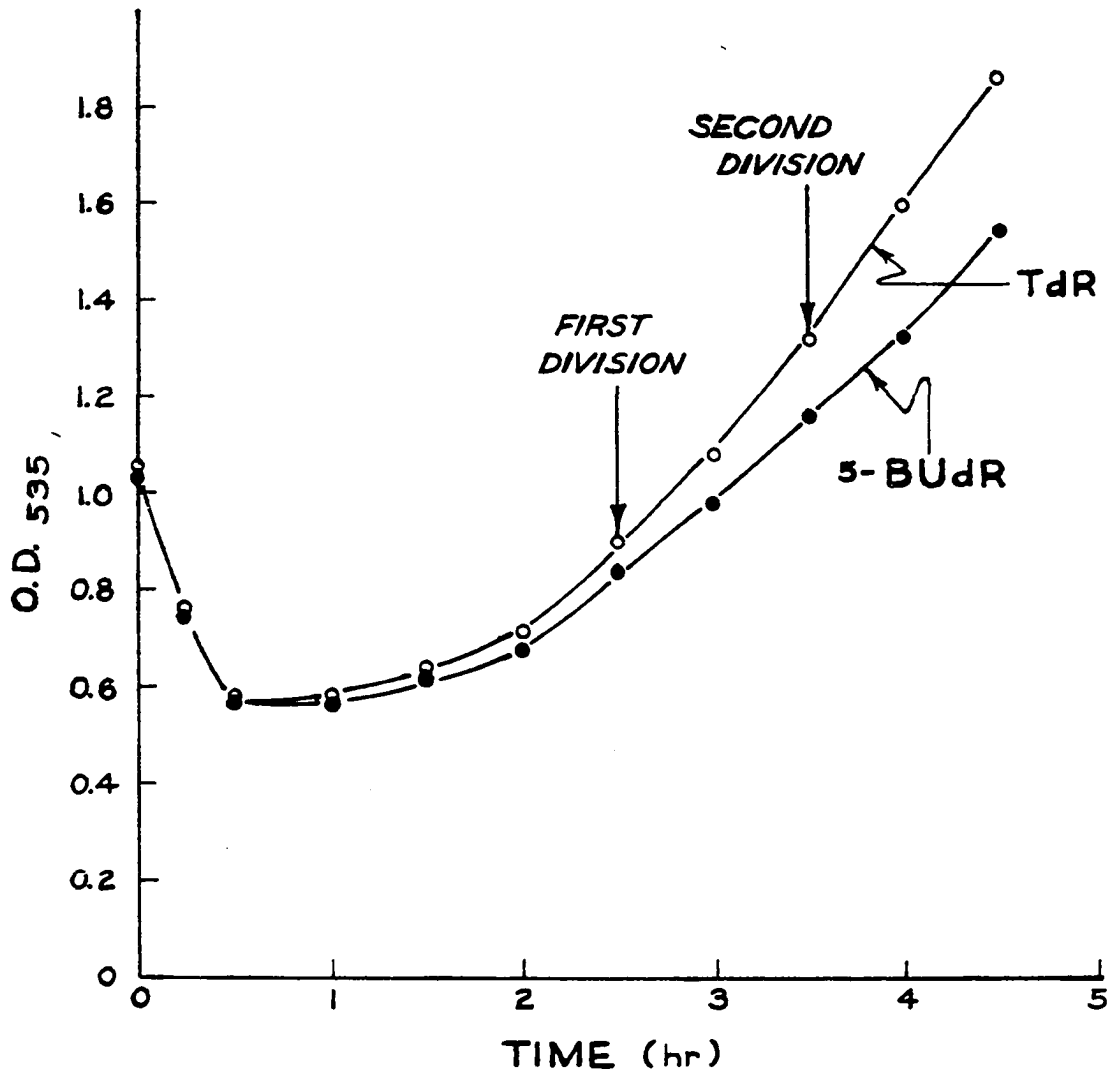


Figure 14. Germination of *B. subtilis* spores. Spores of strain 23 thy-his were germinated in medium containing TdR (o) or 5-BUdR (●). The germinating spores were examined microscopically for the presence of septa to determine the division times. The arrows labeled first and second division are for the TdR culture. No division time could be determined for spores germinating in the presence of 5-BUdR.

### DNA isolation

All DNA samples isolated in the labeling experiments were treated in the same way. After heating the 100 ml samples of germinating spores for 10 min at 60 C to stop DNA replication, the cells were centrifuged at 4 C at 16,000 x g and resuspended in 100 ml of Minimal Medium. The cells were centrifuged again and resuspended in 10 ml 0.2 M BPES, pH 9.0, containing 2.5 mg of lysozyme per ml. The cell suspensions were immediately poured into ZnCl<sub>2</sub>-treated dialysis tubing, 6 mm in diameter. The tubing was suspended in 1.0 M BPES, pH 9.0, in a 1 L graduated cylinder sitting in a 37 C water bath. Lysis was allowed to proceed for 1 hr; the temperature was then raised to 50 C and 1 mg of Pronase per ml was added to the dialysis bag. Pronase was replenished by the addition of 0.5 mg per ml every 12 hr for 3 days. After 3 days the pH of the BPES buffer was adjusted to 7.0 and dialysis continued for 3 more days. The DNA samples were clarified by centrifugation for 15 min in a Sorvall angle-head centrifuge at 4340 x g, then stored at 4 C. The sterility of all DNA samples was ascertained by spreading 0.1 ml aliquots on the surface of TSA plates; no colonies formed after the plates had incubated at 37 C for 24 hr.

### Labeling the origin of the chromosome

This experiment is patterned after the density transfer experiments performed by Sueoka and his coworkers (e.g., Oishi and Sueoka, 1965). They allowed spores of B. subtilis strain 23 thy-his to germinate in the presence of 25 µg of 5-bromouracil per ml and then extracted DNA samples

at various times. The DNA samples were fractionated by CsCl density gradient centrifugation and each fraction was analyzed for transforming activities of different markers. The markers closest to the origin of the chromosome appeared in the hybrid peak first. If all chromosomes in the cell population replicated synchronously, then the location of each marker could be mapped based on the time the marker appeared in the hybrid peak. Two sources of interference complicate the interpretation of the data from density transfer experiments. One is that dichotomous replication occurs, and the other is that synchronous replication of the chromosomes breaks down. By using PEA in the present experiments, it was hoped that dichotomous replication could be inhibited. It has been suggested that PEA prevents the chromosome from entering a new round of replication but allows the chromosome to complete the round of replication already begun at the time of PEA addition (Treick and Konetzka, 1964).

The DNA isolation procedure used by Sueoka and his coworkers is extremely gentle. The cells are treated with lysozyme and sodium lauryl sulfate, and the lysate is centrifuged in a CsCl density gradient. If no chromosomal breakage occurred, one can ask why the newly labeled part of the chromosome moved to the hybrid peak if it was still attached to light DNA. One answer could be that the replication point is a weak point in the chromosome where breakage occurs easily. Another answer could be that the labeled part of the chromosome is sufficiently heavy that it separates from the rest of the chromosome during centrifugation whether there is a replication point or not. If the chromosome labeled

in the origin is allowed to complete replication in light medium, and if shearing of the DNA is kept to a minimum, when the DNA is fractionated by CsCl density gradient centrifugation, the results could suggest which explanation is correct.

It was hoped in this experiment that chromosomes would be labeled at the origin and, after completing the replication cycle in TdR medium so that no replication point existed in the chromosome, the chromosomes could be isolated with minimal breakage using the Massie and Zimm method (1965a). A comparison would then be made of the behavior of the labeled part of the chromosome from partially replicated and fully replicated DNA when centrifuged in a CsCl density gradient.

An outline for this experiment is presented in Figure 15. Ten ml of the B. subtilis strain 23 thy-his spore suspension was added to a Fernbach flask containing 1 L of prewarmed Germination medium plus 100 µg of histidine and 25 µg of 5-BUdR per ml. The flask was shaken at 37 C on a New Brunswick Model VL rotary platform shaker at a setting of "1". At 60, 75, 90, 105, and 120 min, 200 ml were removed and divided into 2 equal parts. One part was heated at 60 C for 10 min to stop DNA replication (Oishi et al., 1964) and the DNA isolated as described. The other 100 ml sample was centrifuged at 4 C at 16,000 x g and resuspended in 100 ml of prewarmed Germination medium containing 0.3% PEA and 25 µg of TdR per ml. The 60, 75, 90, 105, and 120 min samples were shaken at 37 C for 150, 135, 120, 105, and 90 min, respectively, making a total of 210 min of shaking for each sample. Microscopic examination of the PEA-

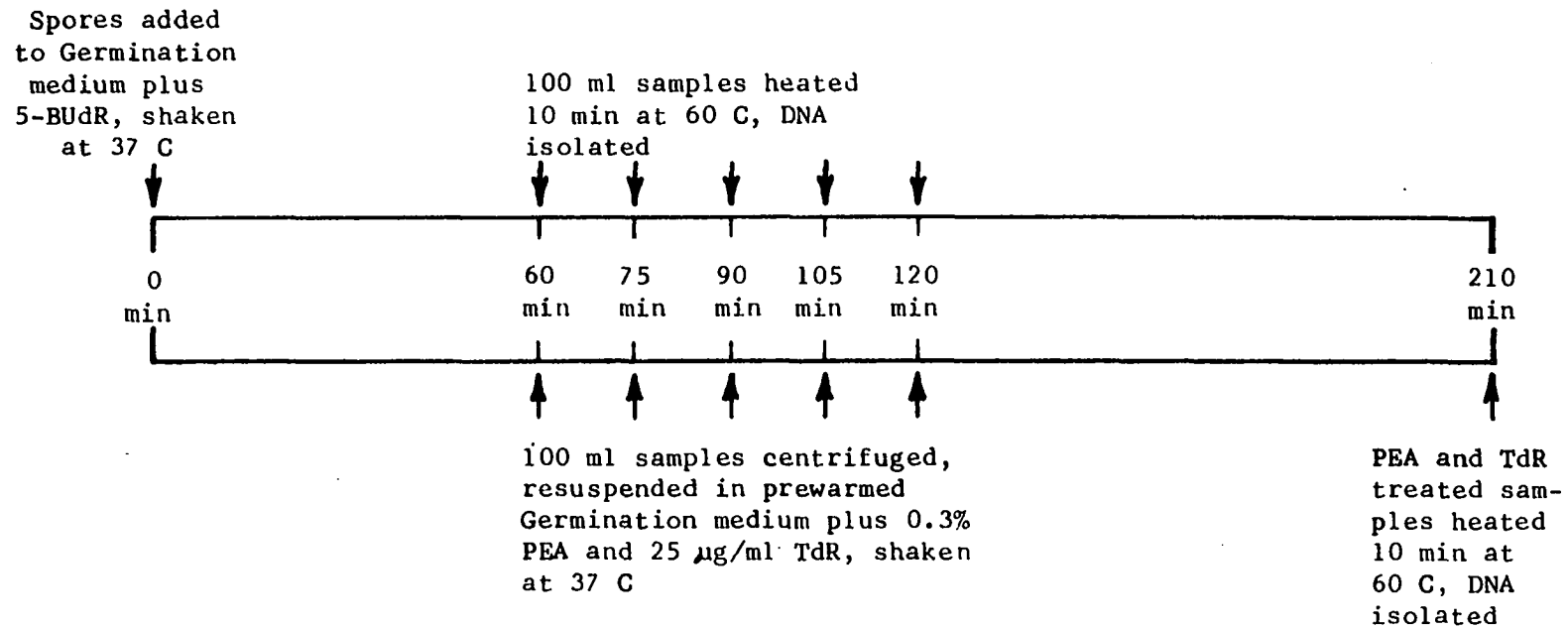


Figure 15. Outline for the experiment labeling the origin of the chromosome.

treated samples after 210 min of shaking revealed cells somewhat uniform in size, approximately 7  $\mu$  in length. No septa were visible. The samples were heated at 60 C for 10 min; the DNA then was isolated. In Table 12, the amounts of DNA isolated from each sample are listed. Also listed in Table 12 are the designations for the DNA samples. In all cases the amount of DNA increased during the PEA treatment, indicating chromosomal replication had taken place; there was, however, never a doubling of the DNA. This result is consistent with the results of Treick and Konetzka (1964).

Table 12. DNA content of DNA samples isolated from germinating spores in the experiment labeling the origin of the chromosome

Treatment of sample	DNA content $\mu\text{g/ml}$
5-BUDR 60 min	17.0
5-BUDR 75 min	20.6
5-BUDR 90 min	18.2
5-BUDR 105 min	22.6
5-BUDR 120 min	21.4
5-BUDR 60 min- PEA and TdR 150 min	29.6
5-BUDR 75 min- PEA and TdR 135 min	29.8
5-BUDR 90 min- PEA and TdR 120 min	28.0
5-BUDR 105 min- PEA and TdR 105 min	27.2
5-BUDR 120 min- PEA and TdR 90 min	30.1

The DNA samples were examined for their ability to transform recipient strain 168 leu-met-adel6. The adel6 marker has been shown to map closest to the origin of the chromosome, while the met marker is closest to the terminus (O'Sullivan and Sueoka, 1967). The leu marker is 0.8 map unit from the origin of the chromosome (Figure 1C). In Table 13, the relative marker frequencies of the adel6 and leu markers for the DNA samples give an indication of the replication pattern in the experiment. The quantitative value of 1.64 for the relative adel6 marker frequency in the DNA sample taken after 120 min in 5-BUdR medium indicates that at least two-thirds of the adel6 markers have replicated in the presence of 5-BUdR and, therefore, should appear in the hybrid peak. PEA did not inhibit dichotomous replication because the 90, 105, and 120 min labeled samples that had been treated with PEA had relative adel6 marker frequencies in excess of 2.0.

The DNA samples were fractionated by CsCl density gradient centrifugation and the fractions analyzed for their ability to transform the adel6, leu, and met markers. O'Sullivan and Sueoka (1967) found that the adel6 was the first marker to appear at the hybrid density of 1.742 g/ml (Szybalski et al., 1960). In Figure 16, after 60 min of exposure to 5-BUdR, a small proportion of the adel6 markers appears in the hybrid peak while the leu and met markers remain exclusively in the light peak at a density of 1.703 g/ml (Szybalski et al., 1960). Figures 17, 18, 19, and 20 show a gradual decrease of the adel6 markers in the light peak until, after 120 min of exposure to 5-BUdR, most of the adel6 markers appear in the hybrid peak.

Table 13. Relative marker frequencies of adel6 and leu markers in DNA samples isolated in the experiment labeling the origin of the chromosome

DNA samples	Experiment <sup>a</sup>	No. transformants x 10 <sup>-1</sup> / 0.1 ml transformation mixture <sup>b</sup>			<u>adel6/met</u>	<u>leu/met</u>	Average		Relative marker frequency	
		<u>adel6</u>	<u>leu</u>	<u>met</u>			<u>adel6/met</u>	<u>leu/met</u>	<u>adel6</u>	<u>leu</u>
DNA sample C	1	2535	3665	3690	.687	.992				
	2	870	1145	1110	.784	1.032	.733	1.017	1.00	1.00
	3	870	1225	1195	.728	1.025				
5-BUdR 60 min	1	1475	1915	1820	.810	1.052				
	2	309	495	428	.722	1.157	.767	1.105	1.05	1.14
5-BUdR 75 min	1	1345	2095	1825	.737	1.150				
	2	349	420	406	.860	1.034	.799	1.092	1.09	1.07
5-BUdR 90 min	1	1830	2535	2140	.855	1.185				
	2	374	498	373	1.003	1.335	.929	1.260	1.27	1.24
5-BUdR 105 min	1	1660	2040	1725	.962	1.183				
	2	456	481	359	1.270	1.340	1.116	1.262	1.52	1.24
5-BUdR 120 min	1	1185	1260	1015	1.167	1.241				
	2	422	424	343	1.230	1.236	1.199	1.239	1.64	1.22
5-BUdR 60 min- PEA and TdR	1	2425	2855	2085	1.163	1.369				
	2	554	569	388	1.428	1.466	1.296	1.418	1.77	1.39
150 min										

<sup>a</sup>Experiments 1, 2, and 3 refer to an assay of the designated samples with a particular preparation of competent cells.

<sup>b</sup>Each number is a mean of duplicate plate counts.



Table 13. (Continued)

DNA samples	Experi- ment <sup>a</sup>	No. transformants x 10 <sup>-1</sup> / 0.1 ml transformation mixture <sup>b</sup>			<u>ade16/met</u>	<u>leu/met</u>	Average		Relative mark- er frequency	
							<u>ade16/met</u>	<u>leu/met</u>	<u>ade16</u>	<u>leu</u>
		<u>ade16</u>	<u>leu</u>	<u>met</u>						
5-BUdR 75 min- PEA and TdR 135 min	1 2	2110 615	2106 536	1510 362	1.397 1.699	1.430 1.481	1.548	1.456	2.11	1.43
5-BUdR 90 min- PEA and TdR 120 min	1 2	2215 569	2300 548	1300 363	1.704 1.567	1.769 1.501	1.636	1.639	2.23	1.65
5-BUdR 105 min- PEA and TdR 105 min	1 2	2105 623	1760 418	1240 309	1.698 2.016	1.419 1.352	1.857	1.386	2.53	1.36
5-BUdR 120 min- PEA and TdR 90 min	1 2	1585 545	1390 373	820 284	1.933 1.919	1.695 1.313	1.926	1.509	2.63	1.48

Figure 16. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in 5-BUdR medium for 60 min. This DNA sample is referred to in the text as the "5-BUdR 60 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.

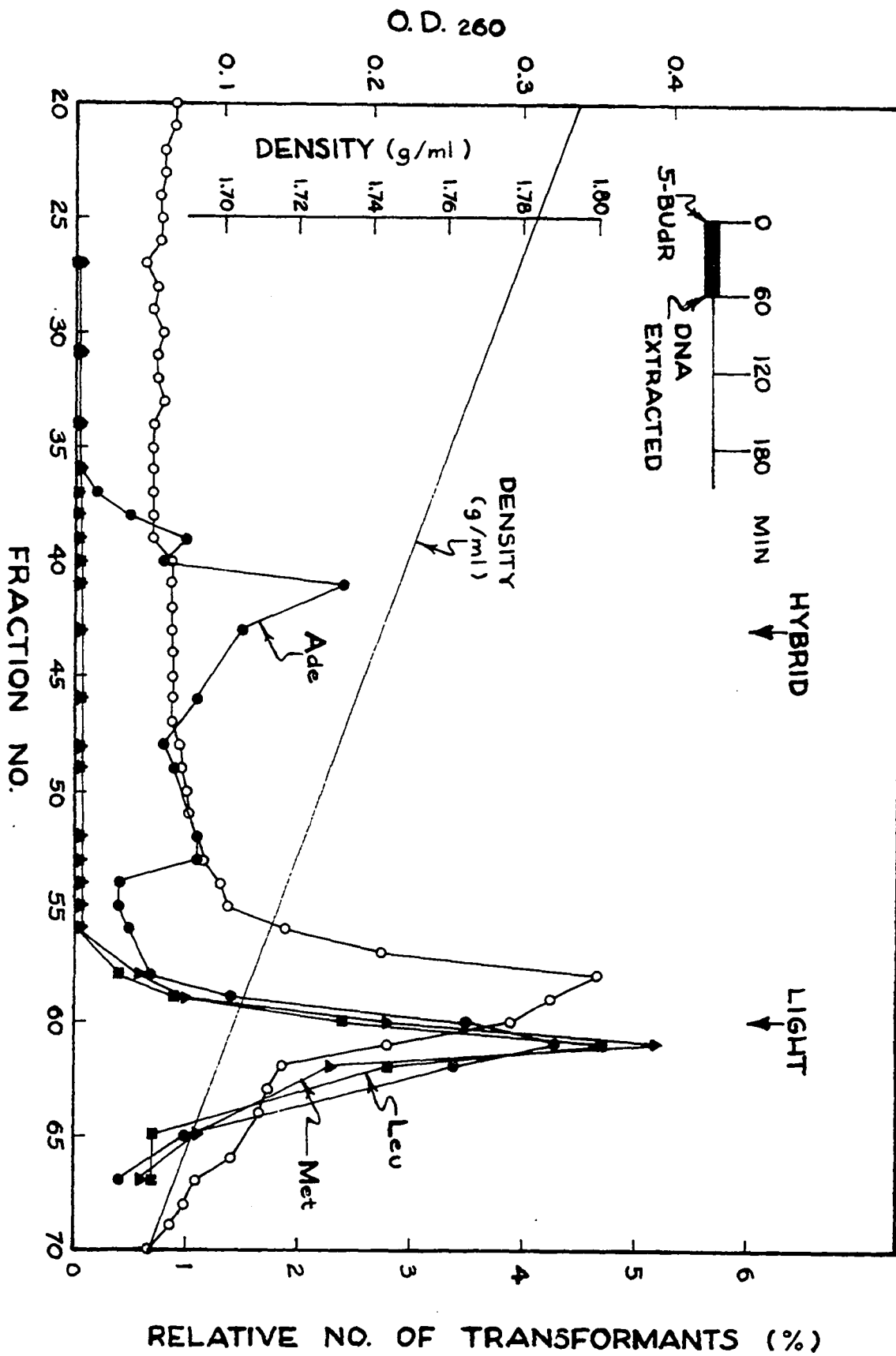


Figure 17. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in 5-BUdR medium for 75 min. This DNA sample is referred to in the text as the "5-BUdR 75 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the adel6 (●), leu (■), and met (▲) markers.

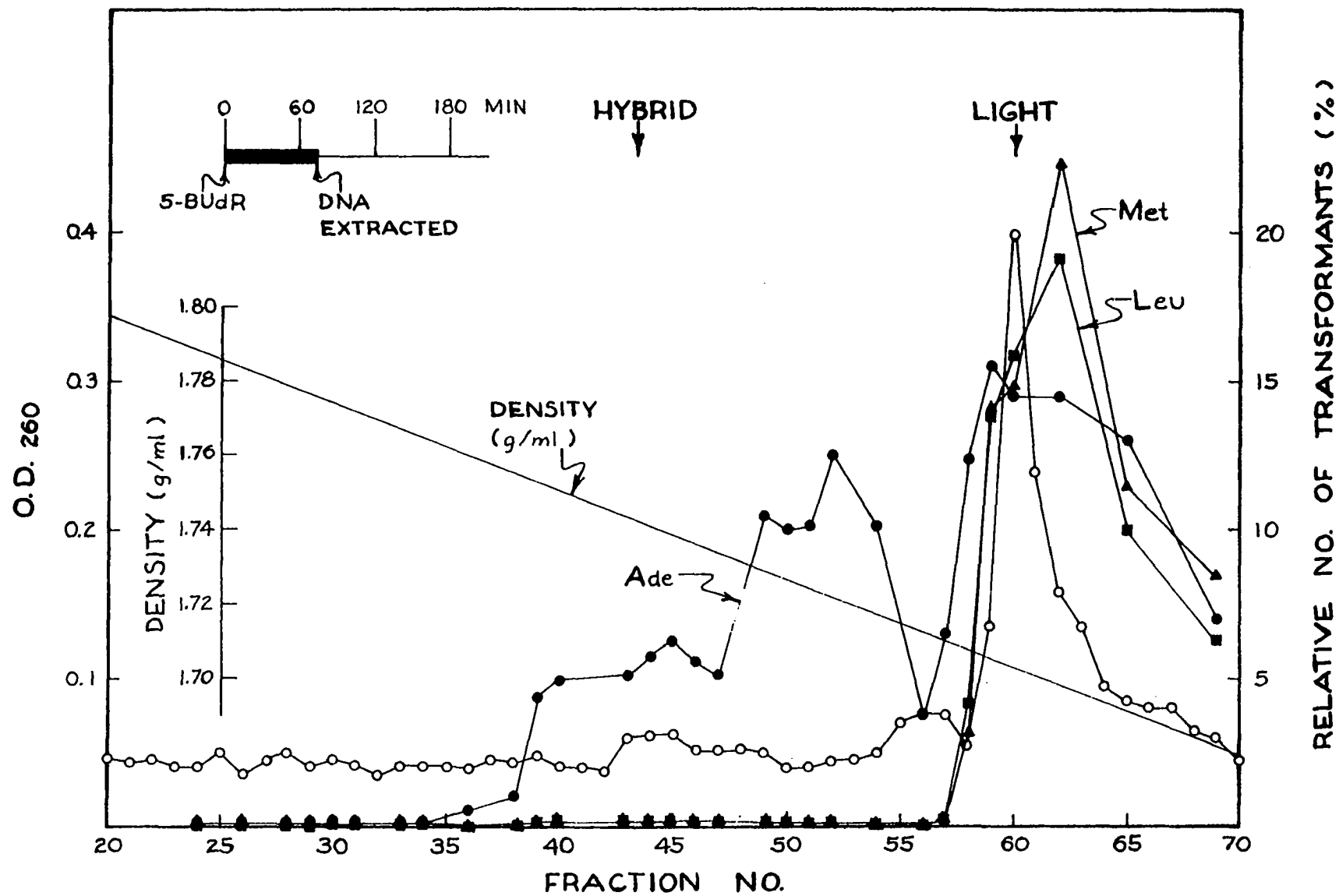


Figure 18. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in 5-BUdR medium for 90 min. This DNA sample is referred to in the text as the "5-BUdR 90 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.

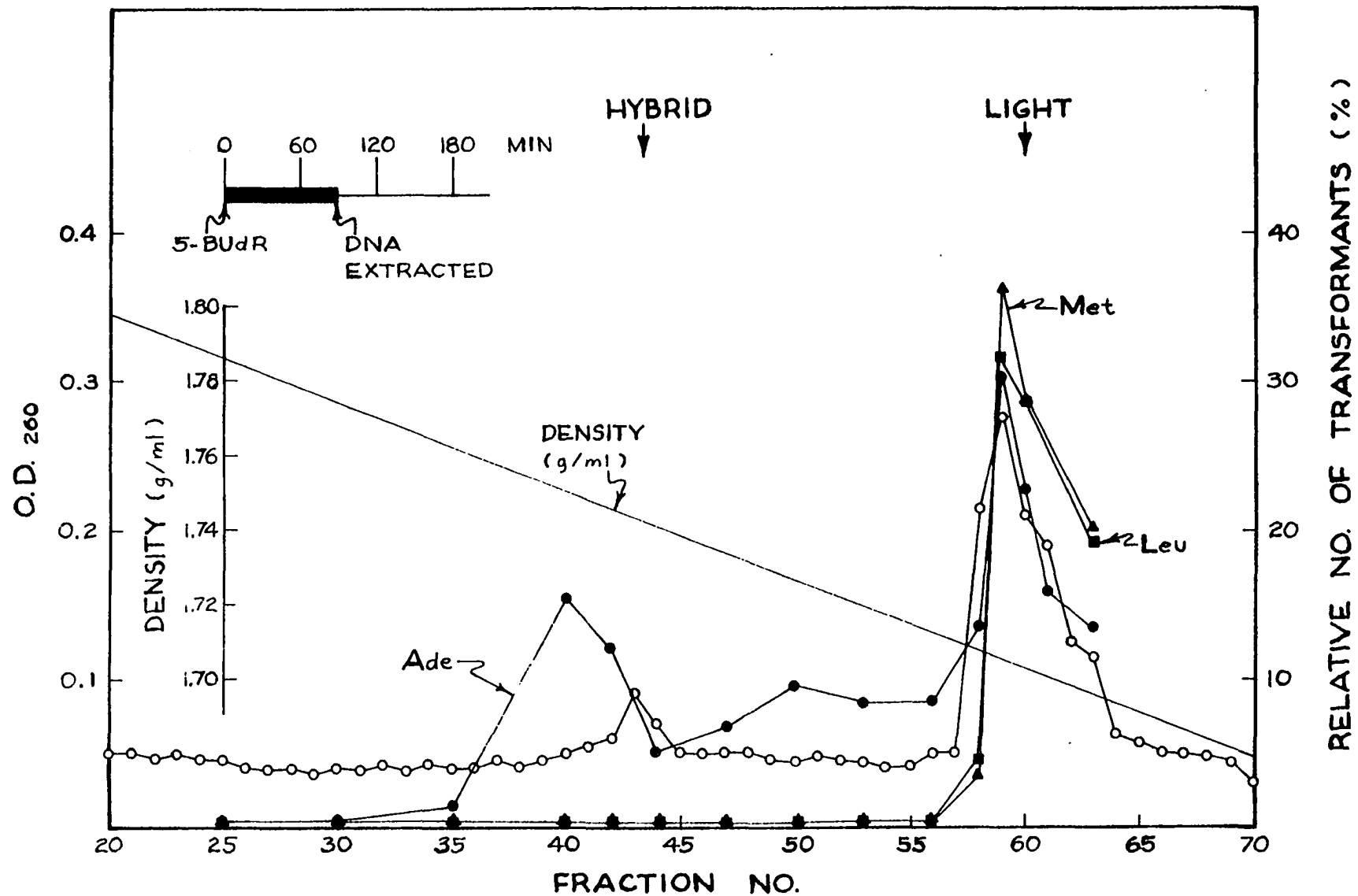


Figure 19. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in 5-BUdR medium for 105 min. This DNA sample is referred to in the text as the "5-BUdR 105 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.



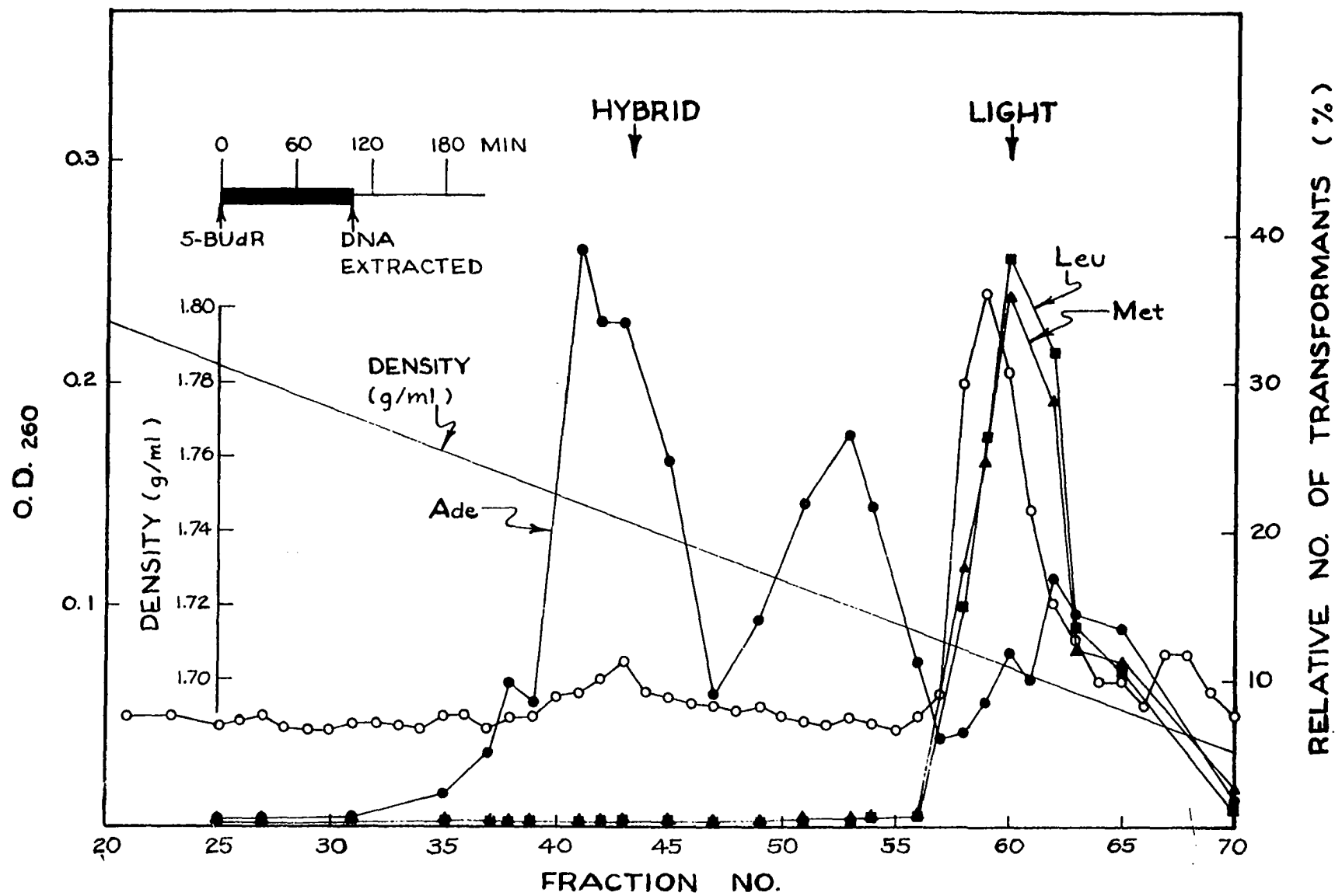
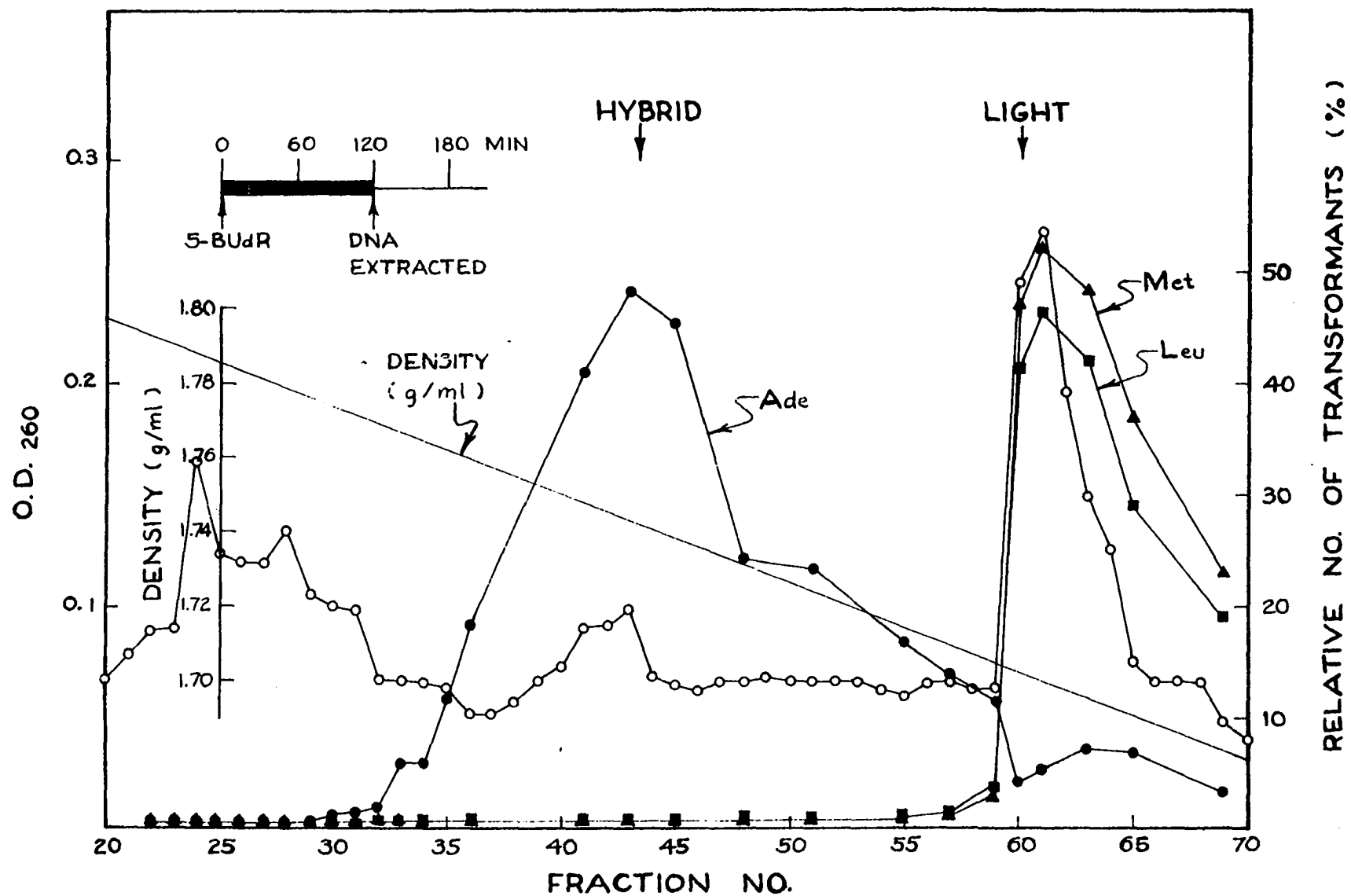


Figure 20. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in 5-BUdR medium for 120 min. This DNA sample is referred to in the text as the "5-BUdR 120 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.

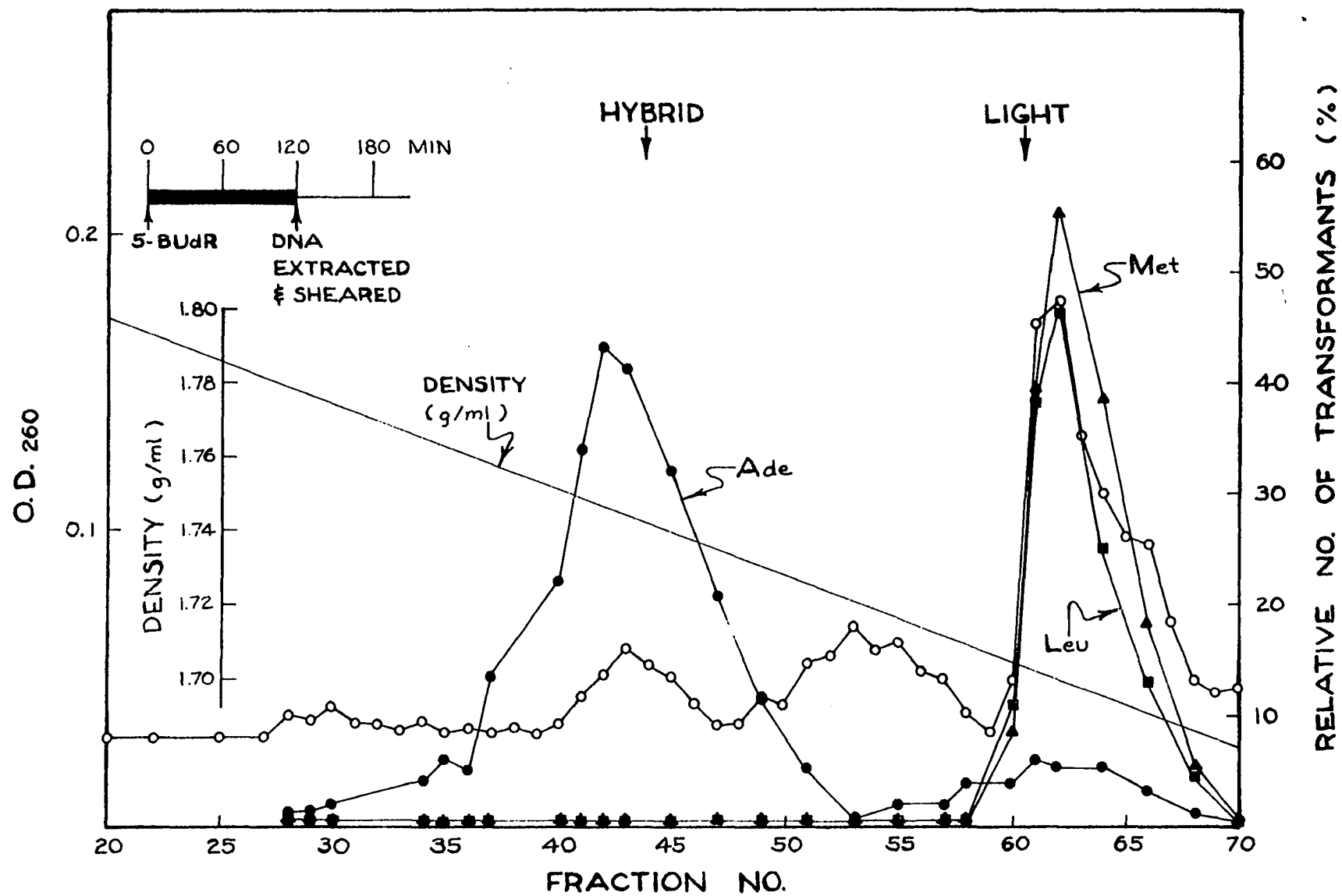


The "5-BuDR 120 min" DNA sample was sheared through a syringe needle and then fractionated by CsCl density gradient centrifugation. The results seen in Figure 21 showed that the DNA activities, as measured by O.D. and transformation frequencies, were located at the same densities as the DNA activities of the unsheared "5-BuDR 120 min" DNA sample (Figure 20). The only difference was that, in the CsCl density gradient centrifugation of the unsheared sample, the adcl6 markers formed a more broad peak at the hybrid density.

If all of the adcl6 markers had been replicated once and none of the met markers had been replicated in a DNA sample, then the relative adcl6 marker frequency value of that sample would be 2.0. The CsCl density gradient centrifugation of the sheared and unsheared "5-BuDR 120 min" DNA sample showed that most of the chromosomes had replicated the adcl6 marker once, but had not replicated the met marker; the adcl6 markers appeared almost exclusively at the hybrid density and the met markers were found only at the light density. Therefore, the relative adcl6 marker frequency value of the "5-BuDR 120 min" DNA sample should be 2.0. The actual value was 1.64 (Table 13), indicating that the 5-BuDR incorporated into the origin region of the chromosome might be depressing the transformation frequency of the adcl6 marker.

The O.D. peak seen in the CsCl density gradient centrifugation of the DNA samples should indicate the density of the majority of the DNA molecules present in the sample. The O.D. peaks in the CsCl density gradient centrifugation of the "5-BuDR 60 min", "5-BuDR 75 min", "5-BuDR

Figure 21. CsCl density gradient centrifugation fractionation of the sheared "5-BUdR 120 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.



90 min", "5-BUdR 105 min", "5-BUdR 120 min", "5-BUdR 60 min-PEA and TdR 150, and "5-BUdR 75 min-PEA and TdR 135 min" DNA samples are all located close to the light density of 1.703 g/ml. In the CsCl density gradient centrifugation patterns of the "5-BUdR 90 min-PEA and TdR 120 min", "5-BUdR 105 min-PEA and TdR 105 min", and "5-BUdR 120 min-PEA and TdR 90 min" DNA samples, the O.D. peaks are located at intermediate densities between the light density of 1.703 g/ml and the hybrid density of 1.742 g/ml.

A comparison of Figures 16, 17, 22, and 23 shows that the adel6 markers seen in the hybrid peaks of the "5-BUdR 60 min" (Figure 16) and "5-BUdR 75 min" (Figure 17) DNA samples are not present in the CsCl density gradient centrifugation patterns of the "5-BUdR 60 min-PEA and TdR 150 min" (Figure 22) and "5-BUdR 75 min-PEA and TdR 135 min" (Figure 23) DNA samples. If the replication point is a weak point in the chromosome and during DNA isolation or CsCl density gradient centrifugation the chromosome breaks at the replication point, this would explain why the adel6 markers are found in the hybrid peak of the fractionated "5-BUdR 60 min" and "5-BUdR 75 min" DNA samples. When the chromosome is allowed to complete replication in the presence of PEA and TdR so that there is no replication point in the chromosome, the markers which had been labeled prior to the PEA and TdR treatment should not separate into the hybrid peak upon centrifugation in a CsCl density gradient. This is what appears to have occurred in Figures 22 and 23 because there are no adel6 markers located at the hybrid density.

Figure 22. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in 5-BUdR medium for 60 min, then in PEA and TdR containing medium for 150 min. This DNA sample is referred to in the text as "5-BUdR 60 min-PEA and TdR 150 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.



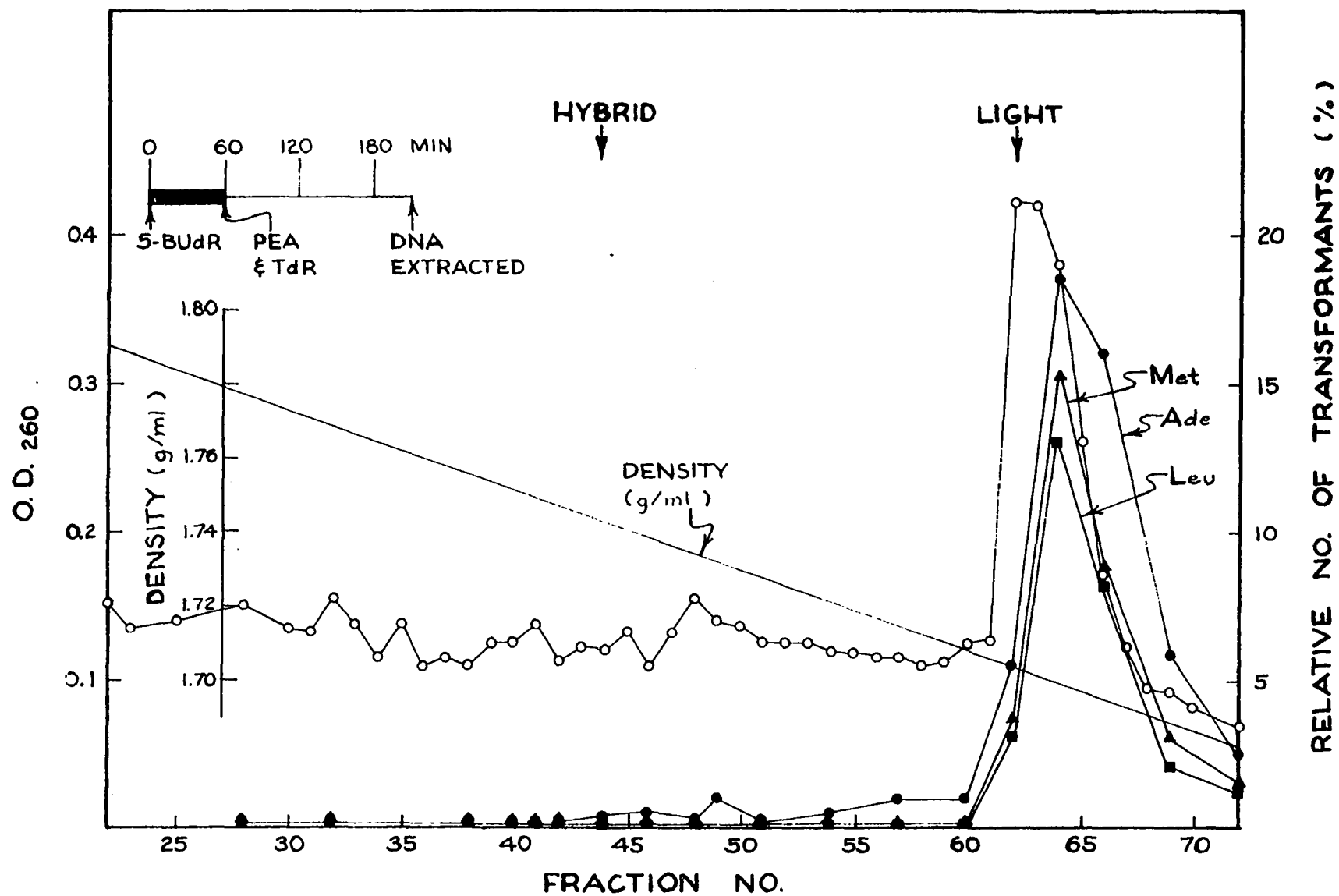
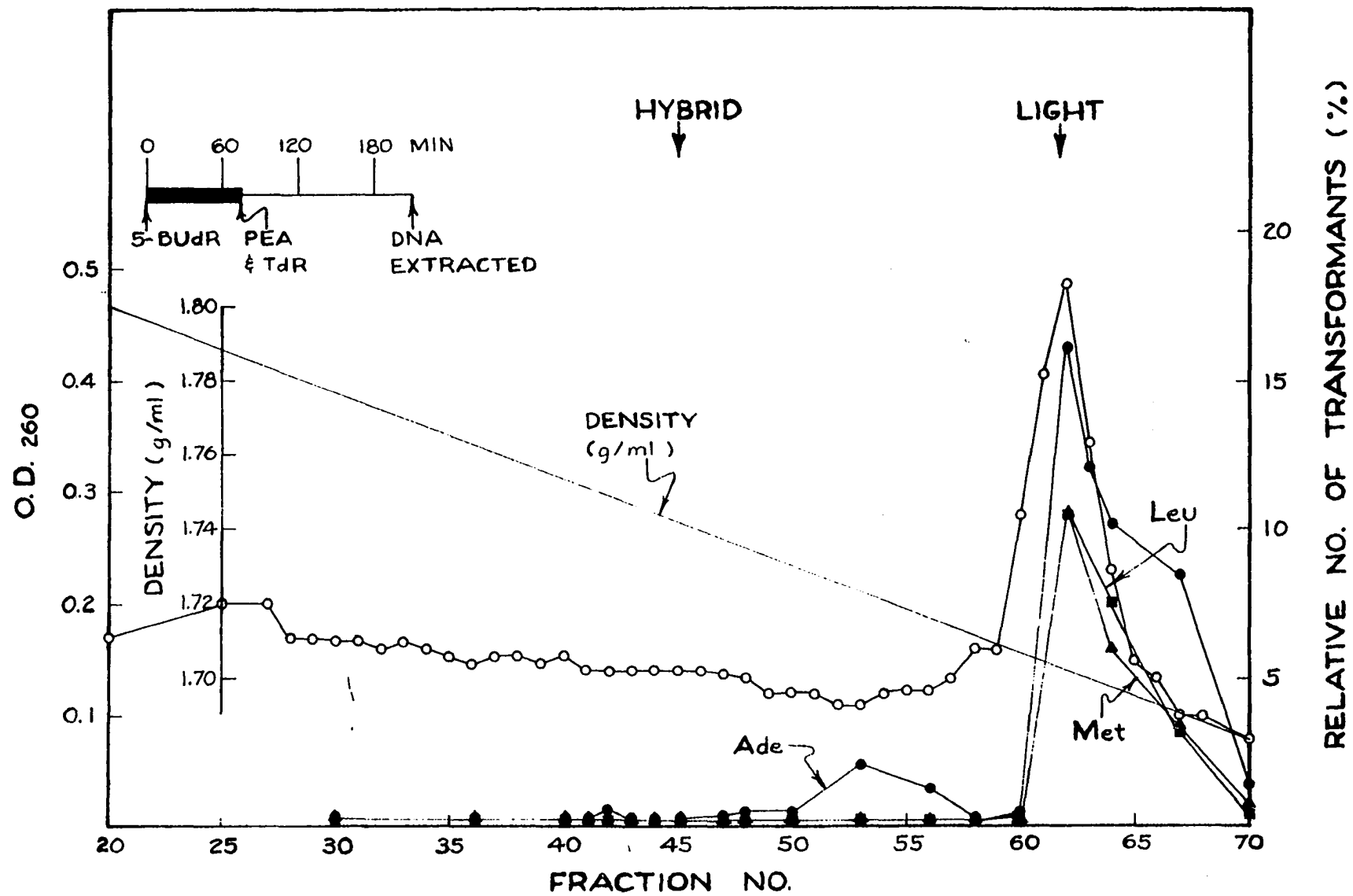


Figure 23. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in 5-BUdR medium for 75 min, then in PEA and TdR containing medium for 135 min. This DNA sample is referred to in the text as "5-BUdR 75 min-PEA and TdR 135 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.



If enough 5-BuDR is incorporated into a portion of the chromosome and the labeled part of the chromosome remains attached during centrifugation, then the overall density of the chromosome should be increased. The greater amount of 5-BuDR incorporated, the more dense the chromosome would be. The O.D. peaks are at the light position in the fractionated "5-BuDR 60 min-PEA and TdR 150 min" and "5-BuDR 75 min-PEA and TdR 135 min DNA samples (Figures 22 and 23). This could indicate that not enough 5-BuDR had been incorporated after 60 and 75 min to cause a detectable change in the density of the DNA molecules. In the fractionated "5-BuDR 90 min-PEA and TdR 120 min", "5-BuDR 105 min-PEA and TdR 105 min", and "5-BuDR 120 min-PEA and TdR 90 min" DNA samples (Figures 24, 25, and 26, respectively), however, the density of the majority of the molecules had become heavier because the O.D. peaks are found at intermediate densities between the light density and the hybrid density.

The majority of adel6 markers which were located in the hybrid peak of the fractionated "5-BuDR 90 min" DNA sample (Figure 18) are now found in an intermediate and a light peak of the "5-BuDR 90 min-PEA and TdR 120 min" DNA sample (Figure 24). The intermediate peak between the light and hybrid densities in Figure 24 could represent partially labeled whole chromosomes. The O.D. of the fractions in Figure 24 forms a peak at the same intermediate location as the transforming activities. The main O.D. peak in the CsCl density gradient fractionation of the "5-BuDR 120 min-PEA and TdR 90 min" DNA sample (Figure 26) is in the heaviest position as compared to the peaks formed from all of the other fractionated DNA

Figure 24. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in 5-BUdR medium for 90 min, then in PEA and TdR containing medium for 120 min. This DNA sample is referred to in the text as "5-BUdR 90 min-PEA and TdR 120 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.

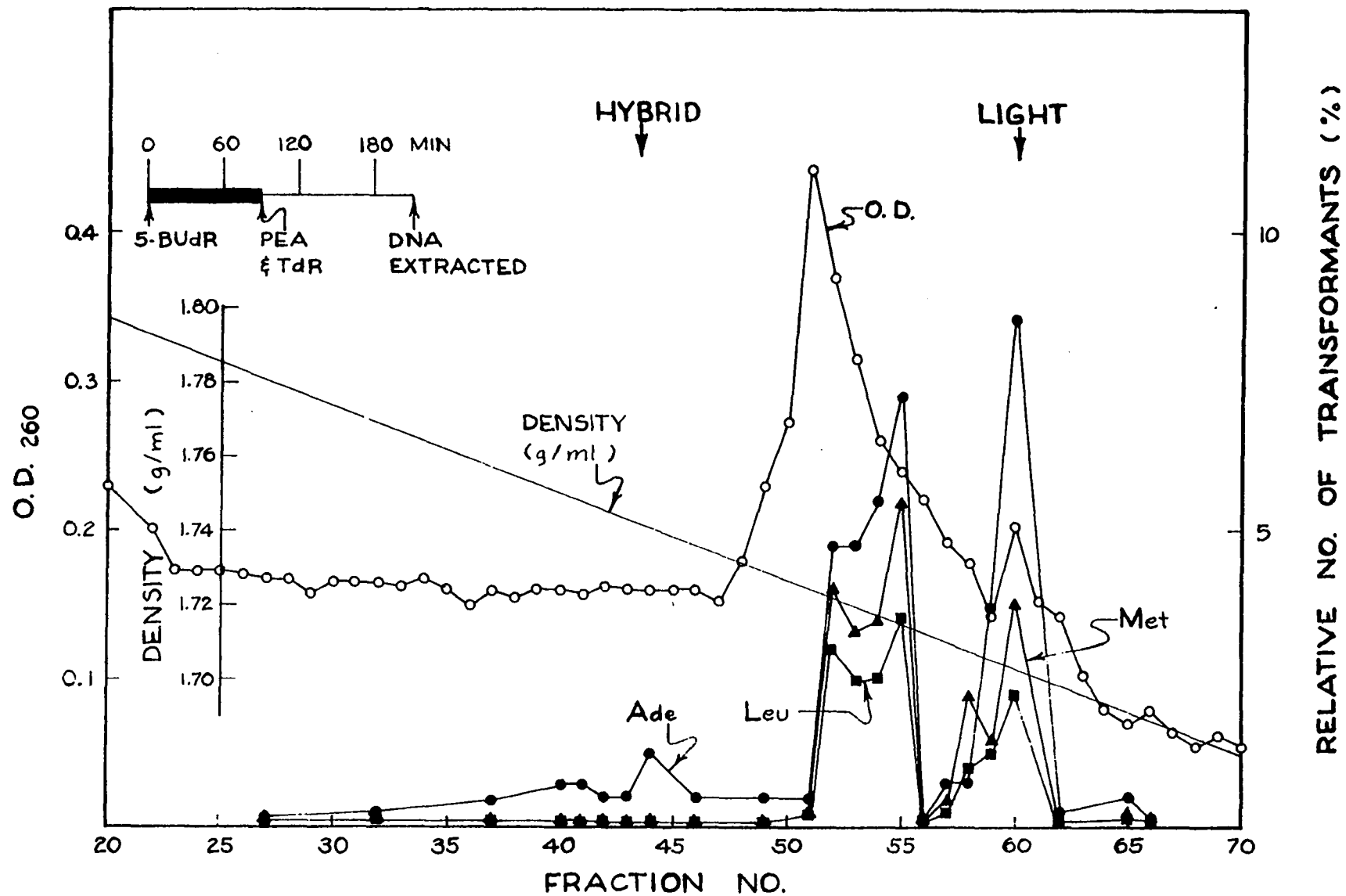


Figure 25. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in 5-BUdR medium for 105 min, then in PEA and TdR containing medium for 105 min. This DNA sample is referred to in the text as "5-BUdR 105 min-PEA and TdR 105 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.

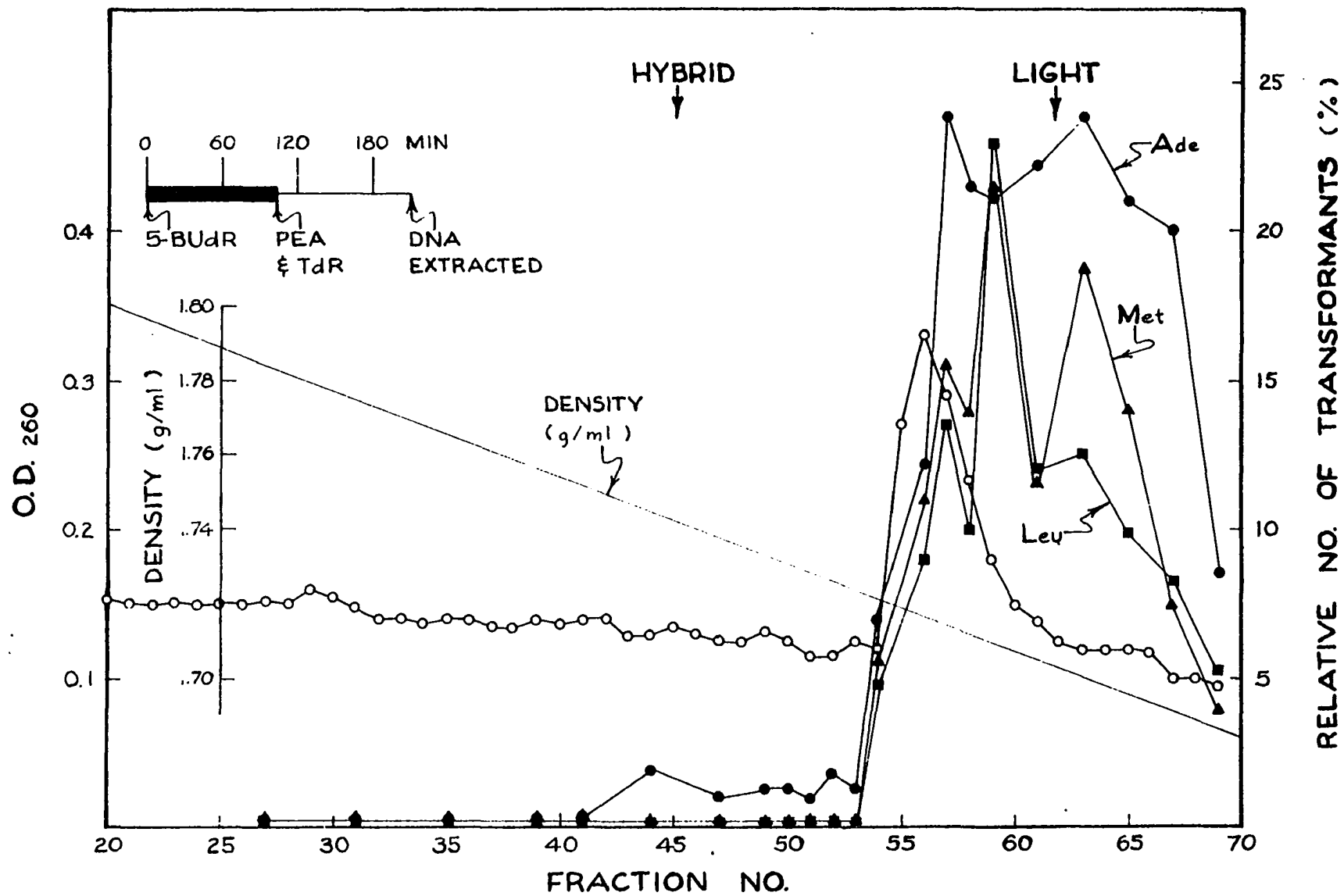
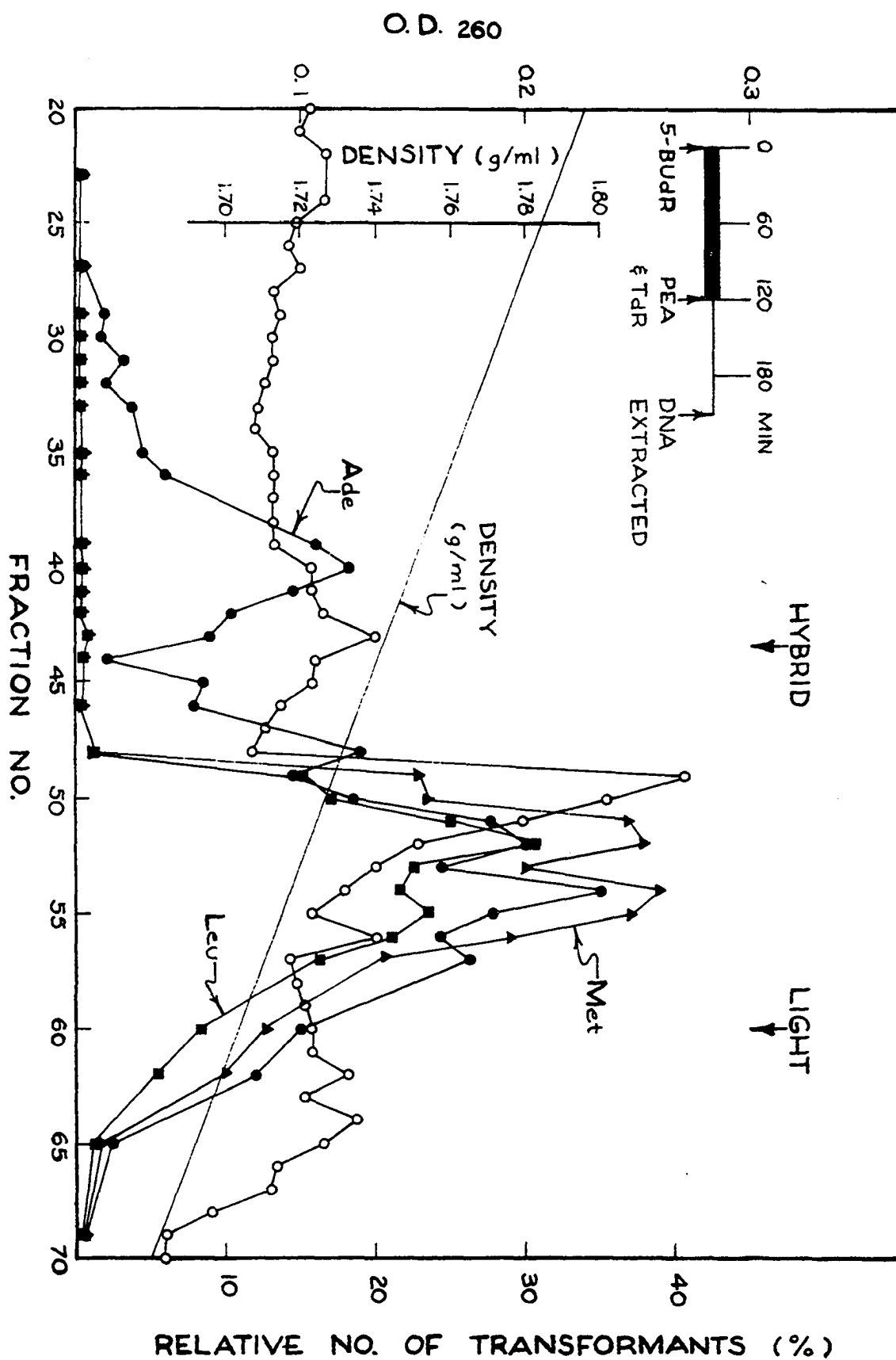




Figure 26. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in 5-BUdR medium for 120 min, then in PEA and TdR containing medium for 90 min. This DNA sample is referred to in the text as "5-BUdR 120 min-PEA and TdR 90 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.



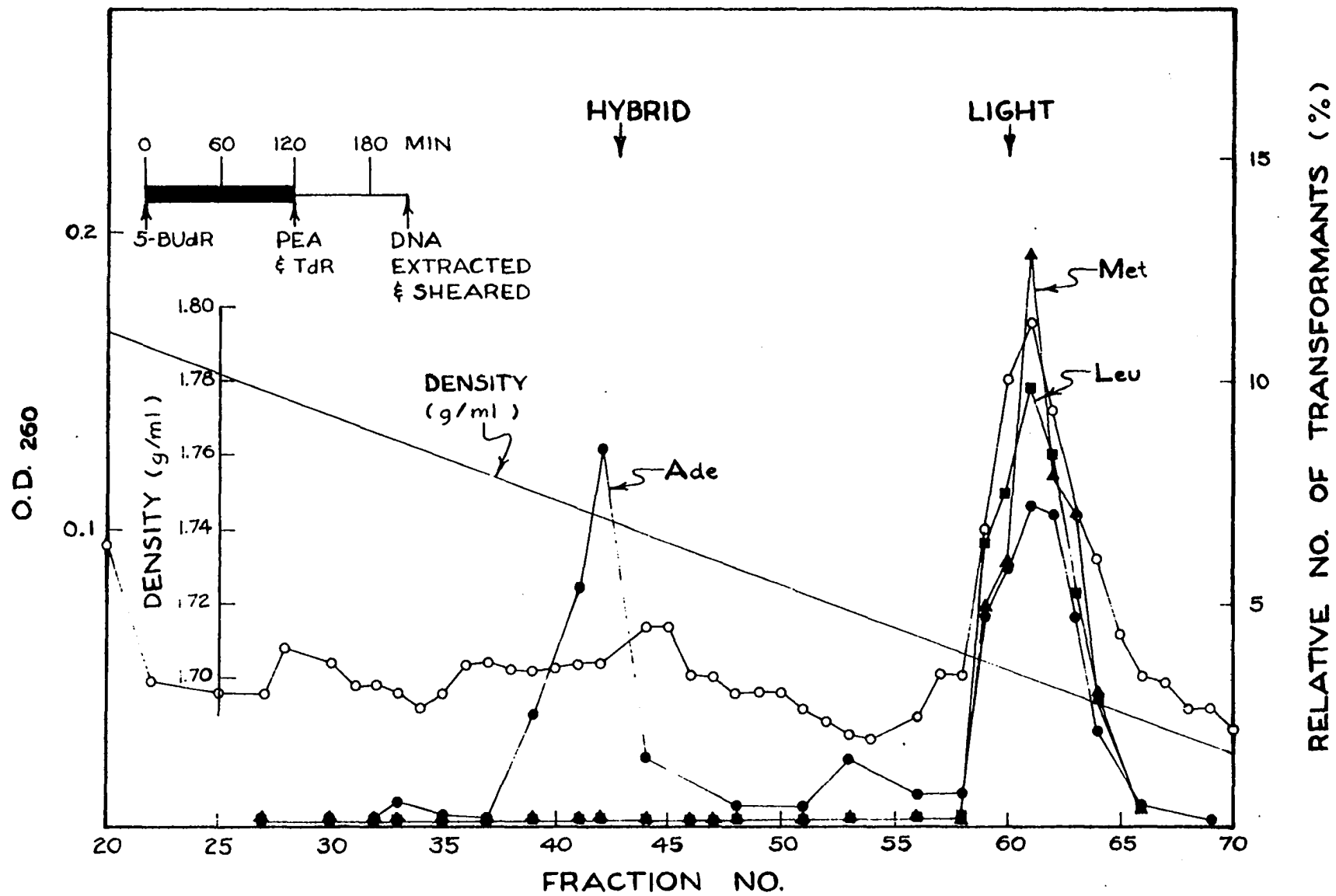
samples in this experiment. The ade16, met, and leu markers are found in fractions between the densities of 1.728 and 1.700 forming a broad peak in Figure 26. There is also a small proportion of ade16 markers located near the hybrid density. The "5-BUDR 120 min-PEA and TdR 90 min" DNA sample was sheared through a syringe needle and then fractionated by CsCl density gradient centrifugation. The results are presented in Figure 27. The intermediate O.D. peak seen in Figure 26 is not present, but there is an O.D. peak at the light density of 1.703. The ade16 marker is evenly divided between the hybrid and light peaks, while the leu and met markers are now found only around the light density.

#### Labeling the terminus of the chromosome

Density transfer experiments have not been useful in determining the replication order of markers at the terminus of the chromosome because synchrony breaks down during the later part of replication (O'Sullivan and Sueoka, 1967). One of the reasons for the deterioration of synchrony may be the presence of 5-BUDR. In this experiment spores germinating in TdR medium were removed and allowed to complete replication in the presence of 5-BUDR. PEA was employed with the hope that it would prevent dichotomous replication but still allow the cells to complete one cycle of replication.

An outline for this experiment is presented in Figure 28. Seven and one half ml of the B. subtilis strain 23 thy-his spore suspension were added to a Fernbach flask containing 750 ml of prewarmed Germination medium plus 100 µg of histidine and 25 µg of TdR per ml. The flask was shaken at 37 C on a New Brunswick Model VL rotary platform shaker at a

Figure 27. CsCl density gradient centrifugation fractionation of the sheared "5-BUdR 120 min-PEA and TdR 90 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.



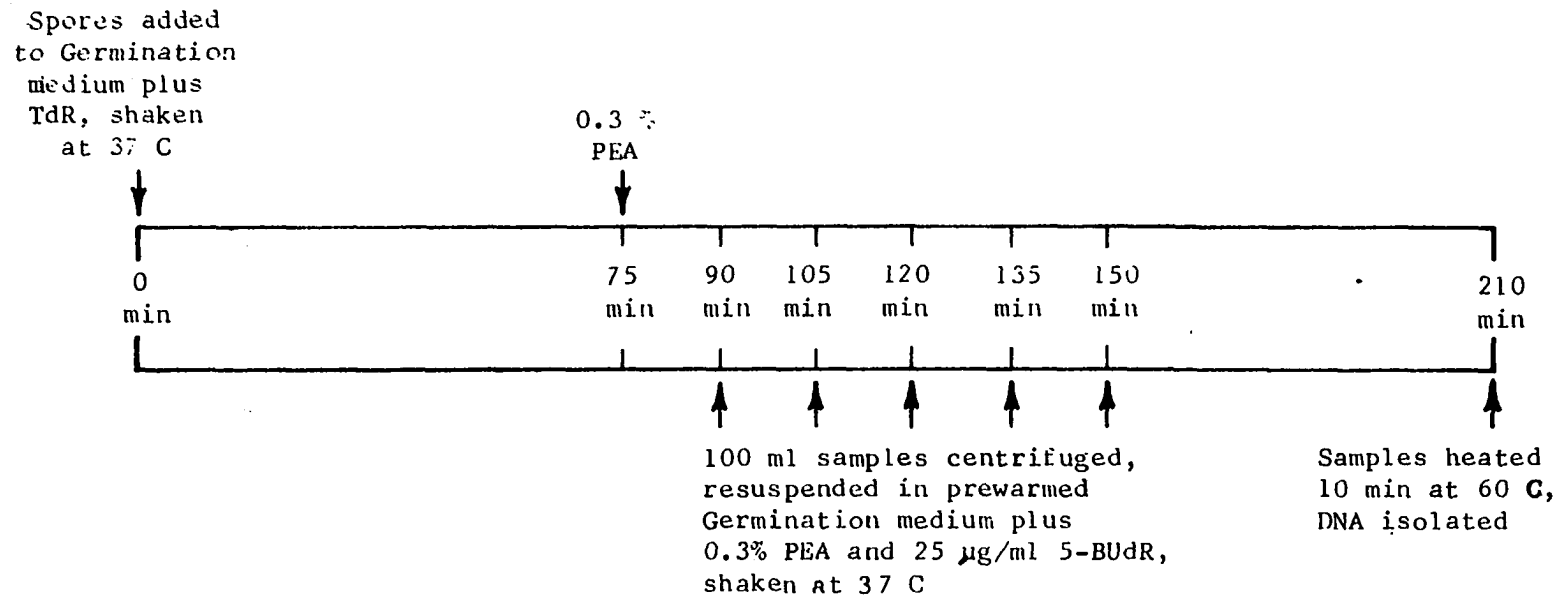


Figure 28. Outline for the experiment labeling the terminus of the chromosome.

setting of "1". After 75 min 0.3% PEA was added and shaking continued. Microscopic examination at 75 min revealed that 70% of the spores had germinated and were approximately  $1.5\ \mu$  in diameter. At 90, 105, 120, 135, and 150 min, 100-ml samples were removed and centrifuged at 4 C at  $16,000 \times g$ . The cells were resuspended in 100 ml of prewarmed Germination medium containing 0.3% PEA and 25  $\mu$ g of 5-BUDR per ml. The 90, 105, 120, 135, and 150 min samples were shaken at 37 C on the rotary platform shaker at a setting of "1" for 120, 105, 90, 75, and 60 min, respectively, making a total of 210 min of shaking for each sample. Microscopic examination at 210 min showed that all of the samples contained cells somewhat uniform in size, approximately  $7.0\ \mu$  in length. The samples were heated for 10 min at 60 C, after which the DNA was isolated. The DNA content of each sample can be found in Table 14. Although the samples had been shaken for the same number of minutes and PEA had been added at the same time, the DNA content of the samples was not the same. The longer the cells were in the presence of TdR, the more DNA was produced, indicating that 5-BUDR was probably inhibiting the rate of DNA synthesis.

The relative marker frequencies of the adel6 and leu markers for the DNA samples are listed in Table 15. The relative adel6 marker frequencies have doubled, and, in two samples, almost tripled the control value, indicating that the adel6 marker has been replicated but also that dichotomous replication has occurred. In the "TdR 90 min-5-BUDR 120 min" DNA sample, the adel6 marker has been replicated as reflected in the

Table 14. DNA content of DNA samples isolated from germinating spores in the experiment labeling the terminus of the chromosome

Treatment of sample (designation)	DNA content μg/ml
TdR 90 min-5-BUdR 120 min	25.2
TdR 105 min-5-BUdR 105 min	27.8
TdR 120 min-5-BUdR 90 min	29.0
TdR 135 min-5-BUdR 75 min	30.0
TdR 150 min-5-BUdR 60 min	32.4

relative ade16 marker frequency value of 2.19. The relative leu-marker frequency value of this sample was 0.99. This 1:1 ratio of leu markers to met markers could be because the leu marker had not been replicated or because the met marker, as well as the leu marker, had been replicated. In the other 4 samples the leu marker had replicated in at least 40% of the chromosomes because the relative leu marker frequency values were higher than 1.40.

The DNA samples were fractionated by CsCl density gradient centrifugation and the O.D. at 260 mμ recorded for each fraction. The results are presented in one graph in Figure 29. None of the DNA peaks were situated at the light or hybrid densities of 1.703 and 1.742 g/ml, but were located at intermediate densities between the light and hybrid densities. The heaviest DNA peaks came from cells exposed to 5-BUdR the longest time, while the lightest DNA peaks were from cells exposed to



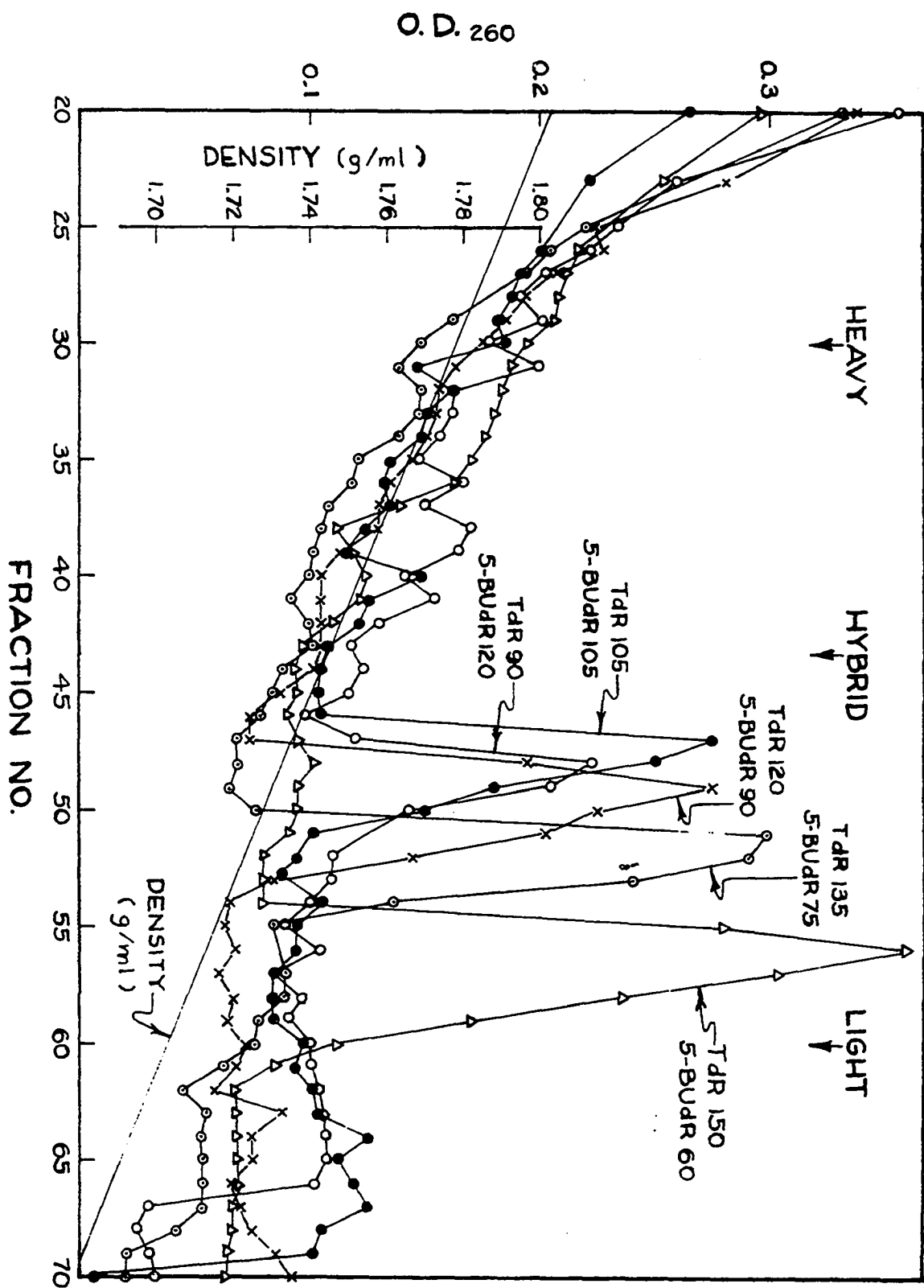
Table 15. Relative marker frequencies of adel6 and leu markers in the DNA samples isolated in the experiment labeling the terminus of the chromosome

DNA samples	Experi- ment <sup>a</sup>	No. transformants x 10 <sup>-1</sup> / 0.1 ml transformation mixture <sup>b</sup>			<u>adel6/met</u>	<u>leu/met</u>	Average		Relative mark- er frequency	
		<u>adel6</u>	<u>leu</u>	<u>met</u>			<u>adel6/met</u>	<u>leu/met</u>	<u>adel6</u>	<u>leu</u>
DNA sample C	1	2535	3665	3690	.687	.993				
	2	870	1145	1110	.787	1.032	.733	1.017	1.00	1.00
	3	870	1225	1195	.728	1.025				
TdR 90 min- 5-BUdR 120 min	2	760	460	570	1.333	.807				
	3	880	570	470	1.870	1.213	1.602	1.010	2.19	0.99
TdR 105 min- 5-BUdR 105 min	2	860	660	380	2.263	1.737				
	3	820	510	410	2.000	1.244	2.137	1.491	2.92	1.47
TdR 120 min- 5-BUdR 90 min	2	950	830	490	1.939	1.694				
	3	850	658	392	2.168	1.679	2.054	1.687	2.80	1.66
TdR 135 min- 5-BUdR 75 min	2	1050	820	520	2.019	1.579				
	3	856	744	464	1.845	1.603	1.932	1.590	2.64	1.56
TdR 150 min- 5-BUdR 60 min	2	1090	920	650	1.676	1.415				
	3	1134	876	606	1.871	1.446	1.779	1.431	2.43	1.41

<sup>a</sup>Experiments 1, 2, and 3 refer to an assay of the designated samples with a particular preparation of competent cells.

<sup>b</sup>Each number is a mean of duplicate plate counts.

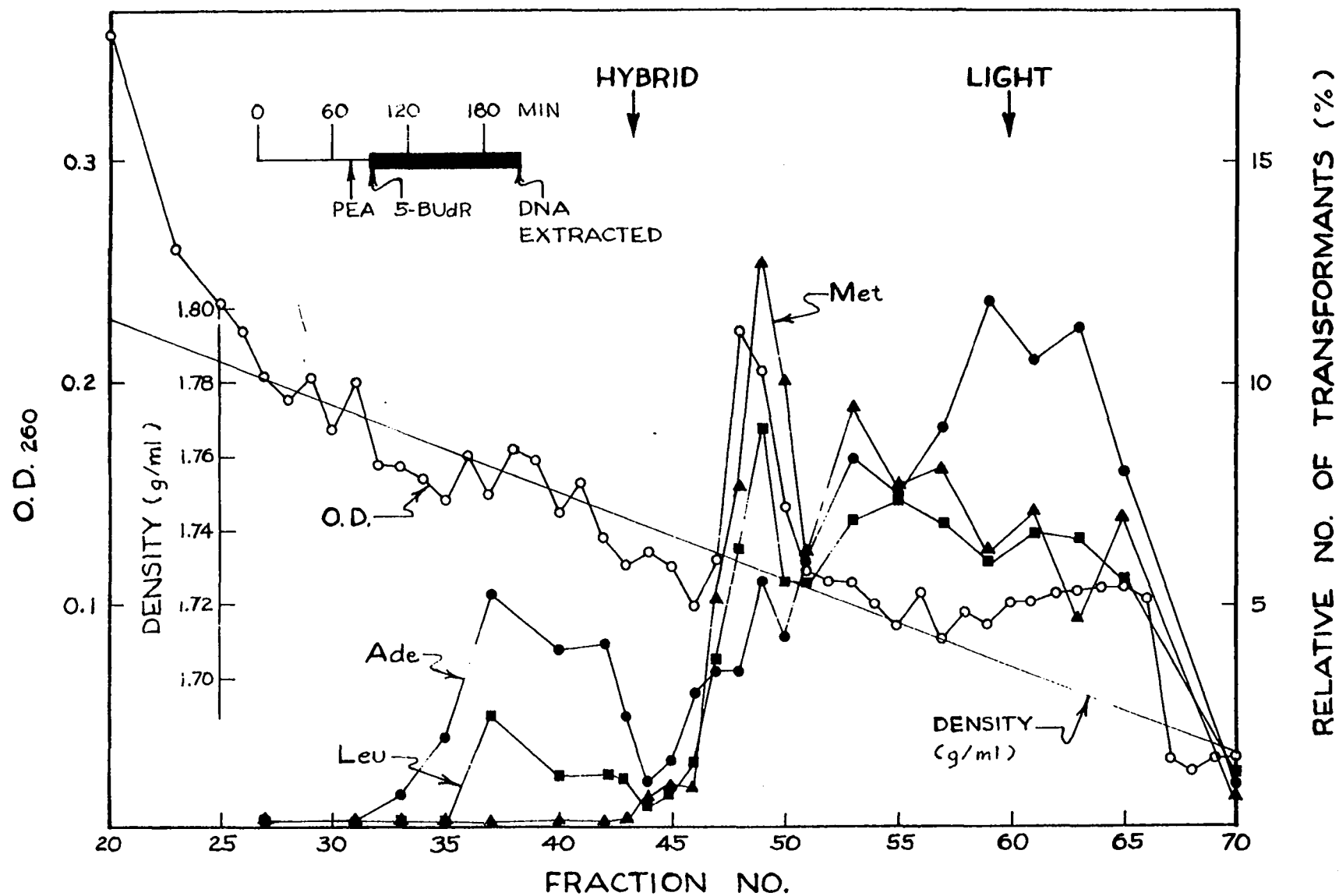
Figure 29. CsCl density gradient centrifugation fractionation of DNA samples isolated in the experiment labeling the terminus of the chromosome. B. subtilis strain 23 thy-his spores were germinated in TdR medium. After 75 min 0.3% PEA was added. Cells were removed at 90, 105, 120, 135, and 150 min, centrifuged, and resuspended in medium containing 5-BUdR and 0.3% PEA. DNA was isolated after a total of 210 min shaking.



5-BUdR the shortest time. When these DNA samples were sheared through a syringe needle and fractionated by CsCl density gradient centrifugation, all of the O.D. peaks returned to the light density of 1.703 g/ml (for examples see Figures 31, 33, and 35).

The fractions from the CsCl density gradient centrifugation of the sheared and unsheared "TdR 90 min-5-BUdR 120 min" "TdR 105 min-5-BUdR 105 min", and "TdR 135 min-5-BUdR 75 min" DNA samples were analyzed for their ability to transform the ade16, leu, and met markers. The transforming activities seen in Figure 30 of the fractions from the "TdR 90 min-5-BUdR 120 min" DNA sample show a broad peak containing all 3 markers between the hybrid (1.742 g/ml) and the light (1.703 g/ml) densities and a small hybrid peak of ade16 and leu markers. Figure 31 shows the results when the CsCl density gradient centrifugation was performed after shearing the "TdR 90 min-5-BUdR 120 min" DNA sample. The transforming activities were no longer in the intermediate broad peak but were located at the hybrid and light densities. The met marker must not have replicated in the 5-BUdR medium, because it is found almost exclusively in the light peak. The leu marker, which maps very close to the met marker near the end of the chromosome (Figure 1C), was evenly divided between the light and hybrid peaks. This indicates that 50% of the chromosomes had almost completed replication. When the unsheared "TdR 90 min-5-BUdR 120 min" DNA sample was centrifuged in a CsCl density gradient (Figure 30), a small proportion of leu markers appeared at the hybrid density, while the majority of leu markers were between light and hybrid densities. When

Figure 30. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in TdR medium for 90 min, then in 5-BUdR for 120 min. PEA was added at 75 min. This DNA sample is referred to in the text as "TdR 90 min-5-BUdR 120 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the adel6 (●), leu (■), and met (▲) markers.



this DNA sample was sheared and then centrifuged in a CsCl density gradient (Figure 31), 50% of the leu markers appeared at the hybrid density and 50% appeared at the light density. A similar situation occurred with the sheared and unsheared "TdR 105 min-5-BUDR 105 min" DNA samples (Figures 32 and 33).

The CsCl density gradient fractionation of the unsheared and sheared "TdR 135 min-5-BUDR 75 min" DNA samples are presented in Figures 34 and 35. There is a peak of O.D. and transforming activity at a density of 1.724 g/ml in the unsheared DNA sample (Figure 34). On shearing, this peak is found at the light density position (Figure 35). Because most of the markers in the intermediate peak of Figure 34 are now located at the light density after shearing (Figure 35), the genetic results do not elucidate whether or not part of the chromosome was labeled with 5-BUDR.

Figure 31. CsCl density gradient centrifugation fractionation of the sheared "TdR 90 min-5-BUdR 120 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.



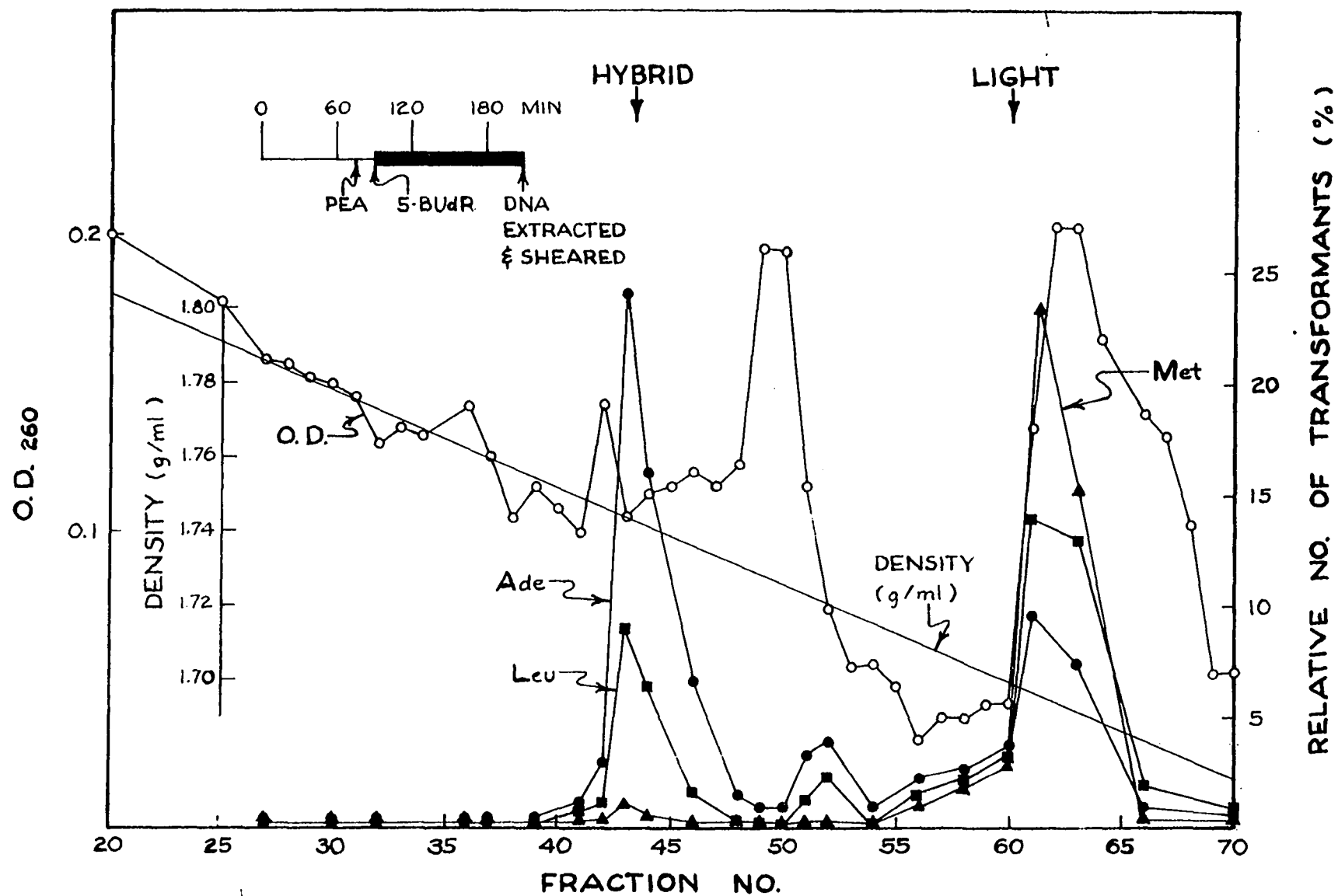


Figure 32. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in TdR medium for 105 min, then in 5-BUdR for 105 min. PEA was added at 75 min. This DNA sample is referred to in the text as "TdR 105 min-5-BUdR 105 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.

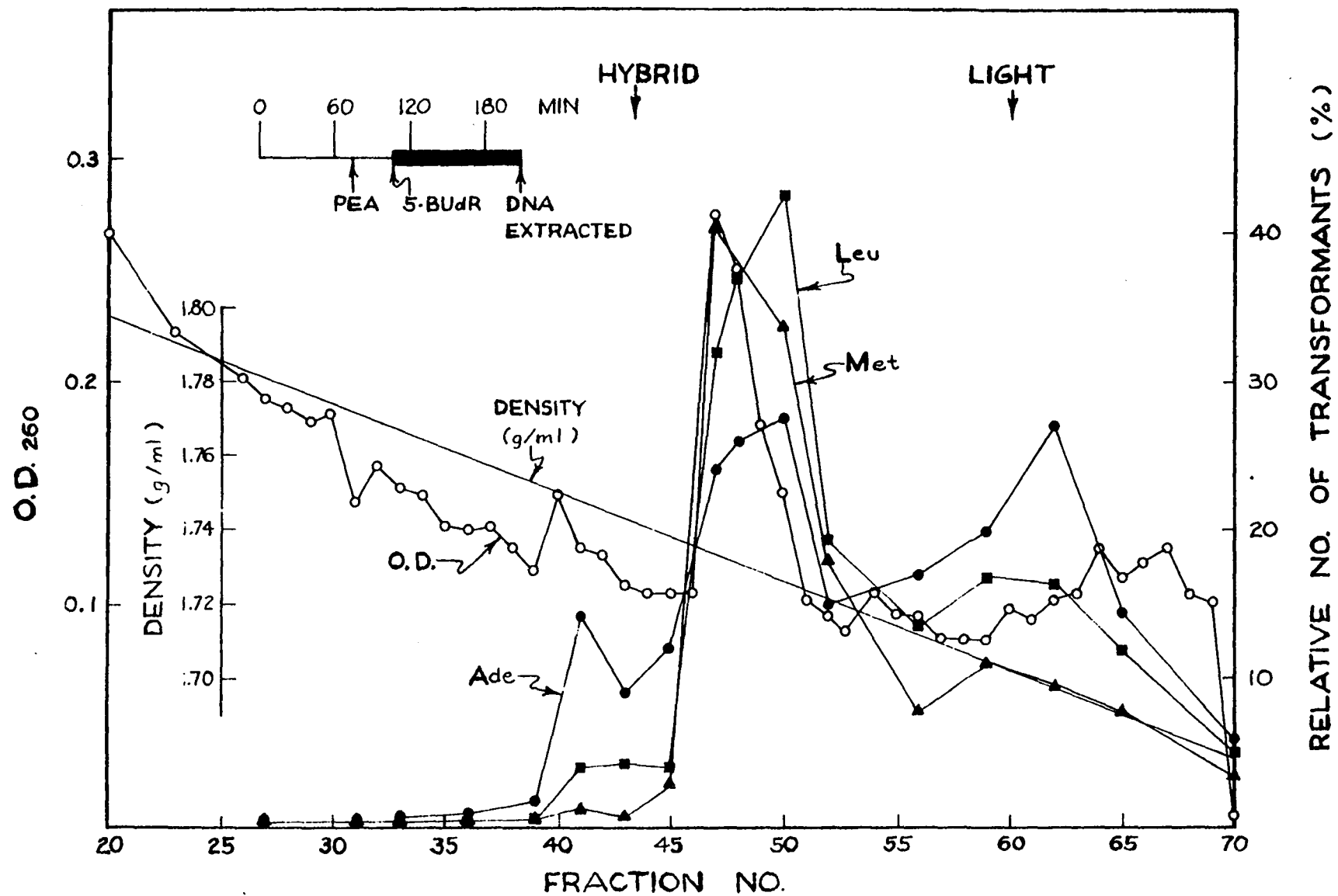


Figure 33. CsCl density gradient centrifugation fractionation of the sheared "TdR105 min-5-BUdR 105 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.

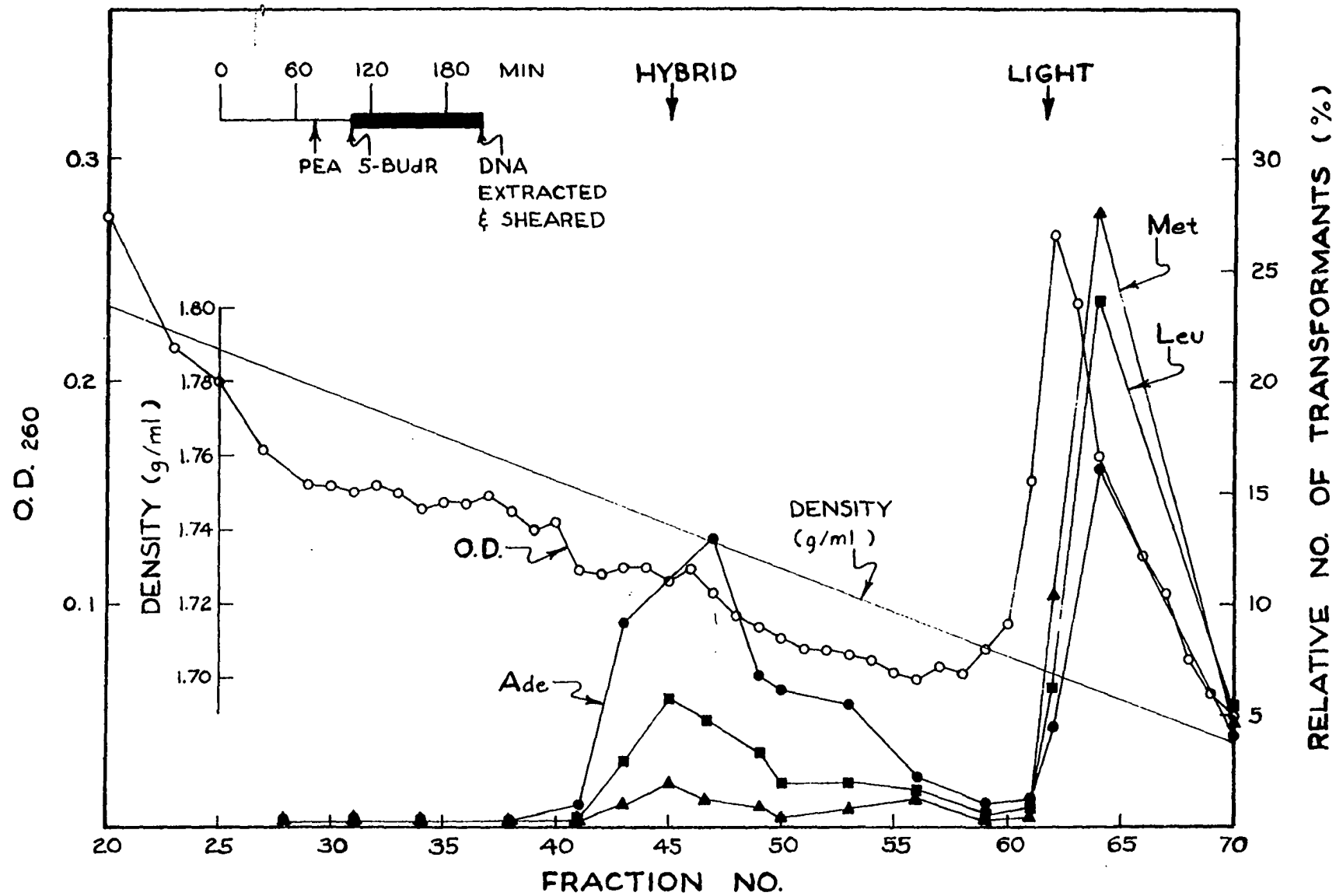


Figure 34. CsCl density gradient centrifugation fractionation of DNA isolated from strain 23 thy-his spores germinated in TdR medium for 135 min, then in 5-BUdR for 75 min. PEA was added at 75 min. This DNA sample is referred to in the text as "TdR 135 min-5-BUdR 75 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.

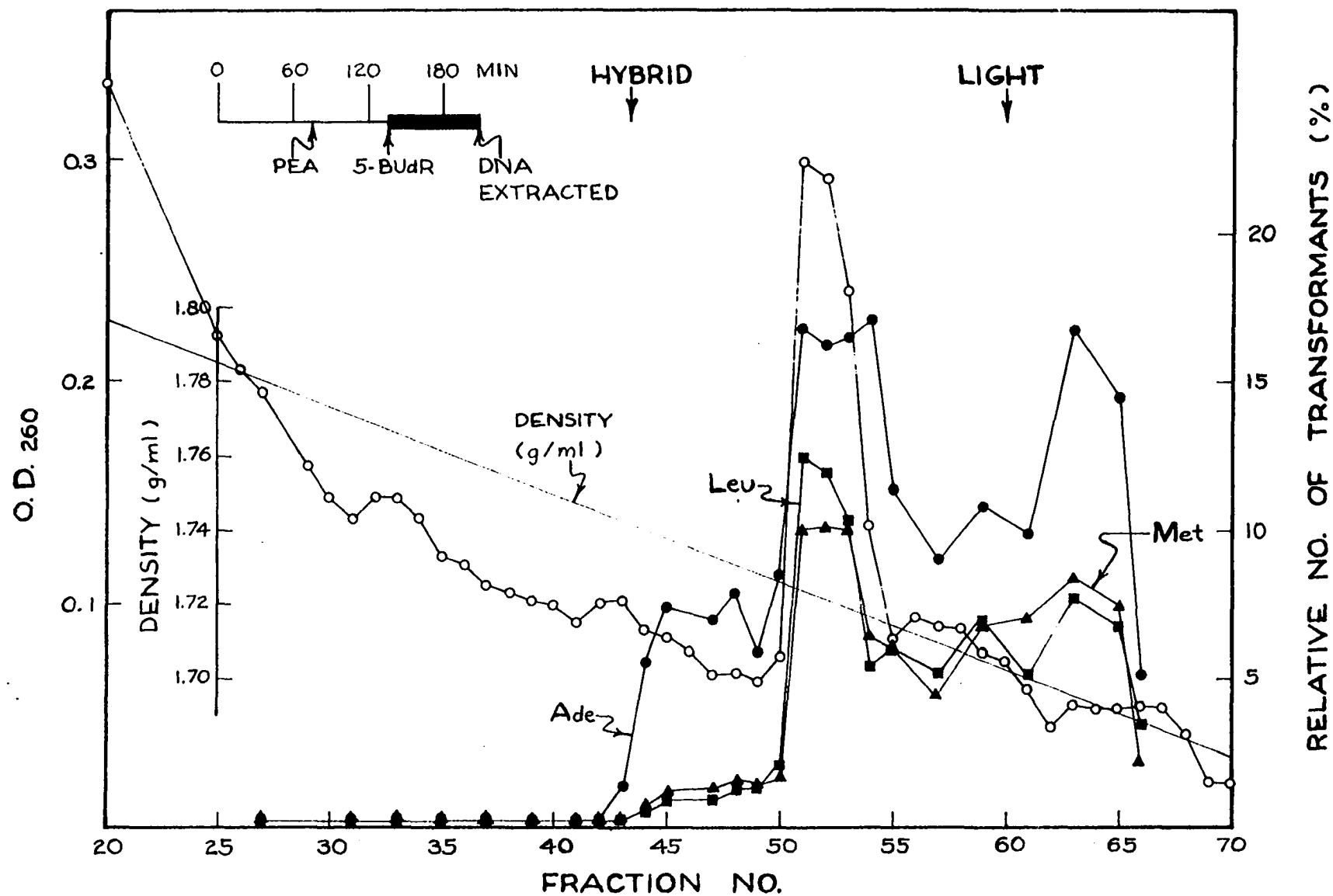
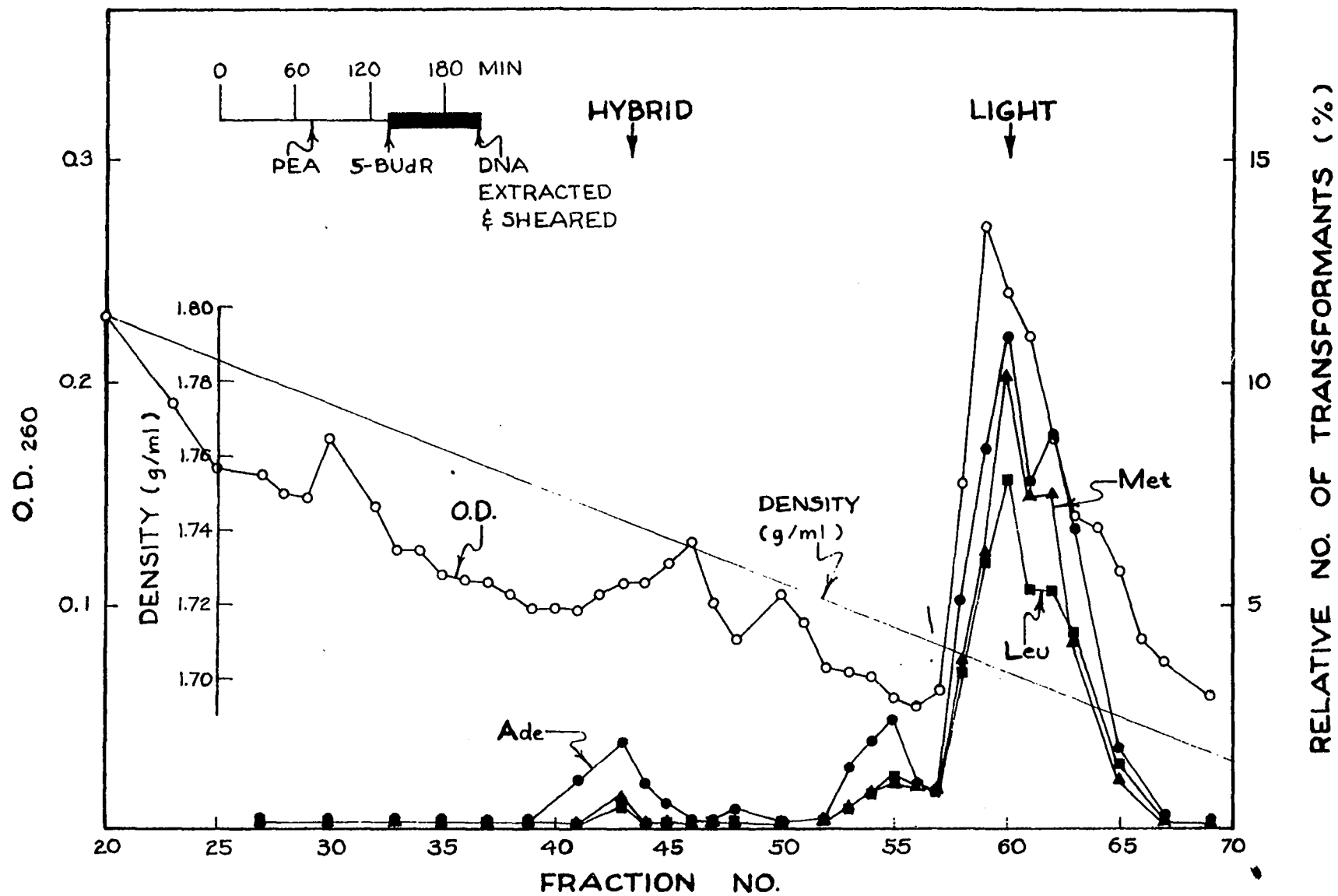


Figure 35. CsCl density gradient centrifugation fractionation of the sheared "TdR 135 min-5-BUdR 75 min" DNA sample. The O.D. at 260 mμ (o) was measured. The fractions were tested for the ability to transform the ade16 (●), leu (■), and met (▲) markers.





## DISCUSSION

The initial object of this study was to determine the existence or nonexistence of protein linkers in the Bacillus subtilis chromosome. By employing the DNA isolation procedure of Massie and Zimm (1965a and b) most of the protein was removed by Pronase and hot phenol. It was hoped that if protein linkers were present they would be removed by this procedure. If the DNA subunits resulting from the removal of protein linkers were different enough physically and chemically then fractionation of the DNA with a MAK column might indicate some separation of the subunits. Saito and Masamune (1964) have claimed that they were able to separate certain genetic marker activities in their study of the fractionation of the DNA of B. subtilis with a MAK column.

The DNA samples which had been treated with Pronase alone (Massie and Zimm, 1965a) had a molecular weight of  $3.05 \times 10^8$  daltons as measured by viscometry and  $2.09 \times 10^8$  daltons as measured by sedimentation velocity studies. These values are in good agreement with the values obtained by Massie and Zimm (1965a) for the molecular weight of the DNA they isolated. There was little chance that the DNA was degraded in any way during the Pronase treatment because the entire procedure was carried out in a dialysis bag and the DNase activity was inhibited with the BPES buffer. When the Pronase-treated DNA samples were treated with hot phenol to remove the remaining protein, viscometry revealed a molecular weight of  $9.99 \times 10^7$  daltons. This is contrary to the results of Massie and Zimm (1965a) who found that the viscosity of the DNA samples did not

appear to change after treatment with hot phenol. Because the phenol-treated DNA samples were found to have a lower molecular weight and because the phenol contaminating the preparation interfered with the ultraviolet light absorption of DNA, DNA preparations were treated only with Pronase to remove the protein. Unfortunately, there was always some protein present in the Pronase-treated DNA preparations.

The experiments with the MAK columns did not reveal whether protein linkers existed in the chromosome. When the unsheared Pronase-treated DNA sample was applied to a MAK column the eluent showed a very broad peak of both genetic activity and absorption beginning at 0.68 M NaCl. The sheared DNA sample started to elute at 0.68 M NaCl, but showed a much sharper elution peak. Both of the DNA samples came from the same container so that the only difference between them was that one was sheared. No marker enrichment could be detected in any of the fractions from the passage of either the unsheared or sheared DNA samples through the MAK column. If any marker enrichment had occurred with the sheared DNA sample and none could be detected for the unsheared DNA sample, this might indicate that DNA isolated by the Massie and Zimm (1965a) procedure contained identical DNA molecules. But, because no marker enrichment occurred with the sheared sample, one can only say that the column did not reveal differences in the population of DNA molecules of both the sheared and unsheared samples. Saito and Masamune (1964), who claimed that certain genetic markers were enriched in restricted fractions when they attempted to fractionate DNA of B. subtilis with a MAK column, il-

illustrated this claim with a graph derived from their data. The curves presented in their graph could have been due to slight fluctuations in the concentration of DNA and due to the fact that most of the recipient strains they employed were mutants of only one locus. It is difficult to make comparisons of marker frequencies when different sets of competent cells are used. This was overcome in these experiments by using recipient strains that were triple auxotrophs.

In the search for techniques that could differentiate between non-identical DNA subunits, a question arose concerning the genetic transfer experiments of Sueoka and his co-workers (e.g., Yoshikawa and Sueoka, 1963b). The DNA isolation procedure used by Sueoka and his co-workers is extremely gentle. The cells are treated with lysozyme and sodium lauryl sulfate, and the lysate is centrifuged in a CsCl density gradient. If no chromosomal breakage occurred, one can ask why the newly labeled parts of the chromosome were found at the hybrid density if it was still attached to light DNA. One answer could be that the replication point is a weak point in the chromosome where breakage occurs easily. Another answer could be that the labeled part of the chromosome is sufficiently heavy that it separates from the rest of the chromosome whether there is a replication point or not. Questions arise regarding the behavior, in the CsCl gradient centrifugation fractionation, of a DNA sample containing partly labeled chromosomes which have no replication points. Would the labeled markers appear at the hybrid density or would they stay attached to the rest of the chromosome? If the labeled markers did not

separate from the rest of the chromosome, would there be a detectable change in the density of the majority of DNA molecules? In order to examine these questions experiments were designed which might produce partly labeled chromosomes containing no replication points.

In the present experiments labeling of the chromosome was accomplished with 5-BUdR rather than 5-BU because of the less toxic effects of 5-BUdR to B. subtilis. The analogue, 5-BUdR, is still toxic and Oishi and Sueoka (1965) have suggested adding TdR to 5-BUdR-containing media in order to decrease this toxicity. In these experiments TdR was not added with 5-BUdR because Laird and Bodmer (1967) have shown that B. subtilis has a marked preference for TdR compared with 5-BUdR. Growth of a B. subtilis thymine auxotroph in a medium containing a mixture of TdR and 5-BUdR could greatly complicate the interpretation of small density differences in the DNA isolated from such a population of cells. The toxic effects of 5-BUdR were seen in this study; 5-BUdR was found to decrease the rate of DNA synthesis and to decrease the rate of transfer of 5-BUdR labeled markers. The locations of the light, hybrid, and heavy densities were based on the values found by Szybalski et al. (1960) for the densities of light, hybrid, and heavy DNA of B. subtilis labeled with 5-BU.

Phenethyl alcohol (PEA) was employed in these experiments because it had been suggested that PEA prevents the chromosome from entering a new round of replication but allows the chromosome to complete the round of replication already begun (Treick and Konetzka, 1964). The concentration of PEA used (0.3%) was selected because it was found to be bacterio-

static (the viable cell count remained constant for 2 hr after the addition of 0.3% PEA) and because DNA synthesis still continued in the presence of 0.3% PEA, repeating the results of Treick and Konetzka (1964). Rosenkranz et al., (1965) on the basis of their results have suggested that the DNA synthesis reported by Treick and Konetzka (1964) to occur after PEA addition was not due to chromosomal replication but rather to the exchange of thymidine in the DNA for the  $H^3$ -thymidine present in the medium. In the present study, though, chromosomal replication did occur in the presence of 0.3% PEA. One source of interference which complicates the interpretation of the data from density transfer experiments is the occurrence of dichotomous replication. Before finishing one round of DNA replication, the chromosome begins a new round of replication. It had been hoped that PEA would inhibit dichotomous replication in these experiments but, in neither the experiments labeling the origin of the chromosome nor the experiments labeling the terminus of the chromosome was this true.

The first step was to duplicate the density transfer experiments of O'Sullivan and Sueoka (1967). Using the technique of transformation after density transfer experiments these investigators were able to construct a genetic map of B. subtilis delineating an origin and terminus. In this study similar results were found with the DNA samples from spores germinated only in 5-BUdR medium in the experiments labeling the origin of the chromosome. In the CsCl density gradient fractionation of these DNA samples the marker closest to the origin, the ade16 marker, appeared

at the hybrid density, while the leu and met markers appeared at the light density. The leu marker is at 0.8 map unit and the met marker is at the terminus of the chromosome (Figure 1C).

In the experiments labeling the origin of the chromosome it was hoped that the first half of the chromosome would be labeled with 5-BUDR, then the chromosome allowed to complete replication in light medium. DNA samples were taken at different times after B. subtilis strain 23 thy-his spores had germinated in 5-BUDR medium. The last sample was taken at 120 min and, if the first division took place at 150 min in 5-BUDR medium as was the case in TdR medium (Figure 14), then 80% of the chromosome would have replicated. All of the chromosomes in these samples should contain a replication point if DNA synthesis had begun in every germinating spore. There would be little chance of contamination of DNA from spores which had not germinated because lysozyme treatment does not lyse spores of B. subtilis. The results of the CsCl density gradient centrifugation fractionation of these samples agreed with the data of O'Sullivan and Sueoka (1967). The ade16 marker which had been replicated in these samples moved to the hybrid peak. Dichotomous replication had not occurred in any of the samples because none of the ade16 markers appeared at the heavy density. That the ade16 markers had been replicated is demonstrated in the increased transformation frequency of ade16 markers as compared to met markers between the 60 min sample and the 120 min sample. The ade to met ratio of the DNA sample extracted from cells germinated 120 min in 5-BUDR medium was 1.64 even though most of the ade16 markers appeared at the hybrid density. The presence of

5-BUDR in the labeled markers could be causing a repression of the transformation of these markers. Szybalski et al. (1960) concluded that hybrid or heavy labeling of the transforming DNA with 5-BUDR did not significantly affect the transforming activity for 3 markers. In their experiments, if one of the markers in the DNA preparation had been labeled and another had been unlabeled and if a recipient strain was used which was auxotrophic for all of the markers tested, then comparisons could have been made between the transforming activities of labeled and unlabeled DNA. Because these conditions were not fulfilled in their experiments, it is unlikely that their results demonstrated more than that labeled DNA still contains transforming activity. These conditions were met in the present experiment; the data indicate that the labeling of the ade16 marker with 5-BUDR has decreased the efficiency of transformation of this marker into a recipient strain.

At the same time the DNA samples were extracted from the spores germinating in 5-BUDR medium, transfers were made to PEA- and TdR-containing medium in the hope that the chromosomes would complete replication but not start a new round of replication. When the DNA samples from these cells were centrifuged in a CsCl density gradient most of the hybrid ade16 markers were not found at the hybrid density. In the two DNA samples extracted from cells exposed to 5-BUDR the least amount of time and PEA and TdR the longest amount of time the ade16 markers were located at the light density along with the O.D. peak and the leu and met markers. The 2 DNA samples extracted from cells which had been exposed for the longest amount of time to 5-BUDR and the shortest amount of



time to PEA and TdR showed broad intermediate peaks of genetic activity containing all 3 markers and located between the hybrid and the light densities. The O.D. peaks were also located at the same intermediate densities but were contained in much fewer fractions than the genetic activities. The broad peaks of genetic activity indicate that the DNA samples contained a heterogeneous population of molecules of different densities.

The proportion of adel6 to met markers had increased to 2.63 in the DNA sample extracted from cells transferred at 120 min into PEA and TdR medium. Because the adel6 marker had replicated in most of the cells at 120 min, the increase in the number of adel6 markers in the DNA sample exposed to PEA and TdR was caused by dichotomous replication occurring in some of the chromosomes. The occurrence of dichotomous replication of the adel6 marker indicates that there is a new replication point in the chromosome located somewhere between the adel6 marker and the leu marker. If this new replication point present in some of the chromosomes was also a weak point then a percentage of the adel6 markers should appear at the hybrid peak rather than centrifuging with the rest of the chromosome. If the 5 DNA samples which had been treated with PEA and TdR contained completed chromosomes which were labeled with 5-BUDR at the origin of the chromosome but also contained some chromosomes which had started a new round of replication allowing the chromosome to break at the new replication point, the best separation of these 3 classes of DNA molecules during CsCl density gradient centrifugation fractionation is found in Figure 24. The adel6 markers appearing at the hybrid density

could represent the adel6 markers that had undergone another round of replication. The intermediate peak seen at a density of 1.720 could be composed of chromosomes which had completed one round of replication but had not started another round and which were labeled with 5-BUDR at the origin. All 3 markers are present in this peak in somewhat equal frequencies. It is very possible that whole chromosomes are extracted by the DNA isolation procedure of Massie and Zimm (1965a). When no replication points are present, the chromosomes appear at an intermediate density rather than the hybrid or light densities in this experiment because of the labeled adel6 markers. The light peak could contain the unlabeled chromosomes and the unlabeled parts of chromosomes containing the leu and met markers which had broken off at the new replication point. It would also contain light adel6 resulting from dichotomous replication.

There was a small proportion of the adel6 markers at the hybrid density in the fractionated DNA sample taken from cells grown for 120 min with 5-BUDR and 90 min with PEA and TdR which could have resulted from dichotomous replication. If another round of DNA replication had occurred in every chromosome in these cells then 50% of the adel6 markers should be labeled and 50% should be unlabeled. Also, if the chromosome breaks at the new replication point, then the adel6 markers should be evenly divided between the hybrid and the light peak. Based on these assumptions, the results do not indicate that every chromosome entered a new round of replication because the adel6 marker peak at the hybrid density contains much less than 50% of the total adel6 markers in the

fractionated "5-BUDR 120 min-PEA and TdR 90 min" DNA sample (Figure 26). When this DNA sample was sheared prior to fractionation the majority of DNA molecules appeared at the light peak as evidenced by O.D. (Figure 27). This density change of the DNA peak (O.D.) from 1.720 to 1.703 could have been due to the separation by shearing of the labeled part of the chromosomes from the unlabeled part. After shearing, the number of adel6 markers at the hybrid density increased in proportion to the number of adel6 markers at the hybrid density in the unsheared fractionated DNA sample. This could indicate that the labeled part of the chromosome had been separated from the rest of the chromosome by shearing. The leu and met markers originally seen at the intermediate density between hybrid and light were located at the light density after shearing. Because the leu and met markers were unlabeled before PEA and TdR treatment, they should have remained unlabeled after PEA and TdR treatment, but they did not appear at the light density until the DNA sample was sheared. The adel6 markers appearing at the light density came from another round of replication in the presence of TdR and PEA. Because the explanation of the data is so dependent upon what happens to the adel6 markers during CsCl density gradient centrifugation, the fact that PEA did not inhibit dichotomous replication or rereplication of the chromosome in these experiments interferes with the interpretation of the results. The results do support the following statements. (1) Chromosomal breakage occurs easily at the replication point allowing the labeled part during fractionation of the DNA by CsCl density gradient centrifugation to

appear at the hybrid density (Figures 16, 17, 18, 19, and 20). (2) When the label is still present in the chromosome and the replication point has moved to the end of the chromosome in light media, then the labeled part of the chromosome does not separate into the hybrid peak (Figures 22, 23, 24, 25 and 26). (3) The greater the amount of labeling that is present, the greater is the density of the majority of unsheared DNA molecules. (4) When this partly labeled chromosome containing no replication point is sheared then the labeled markers appear at the hybrid density and the unlabeled markers appear at the light density (Figure 27).

Density transfer experiments have not been successful in determining the replication order of the markers in the terminus of the chromosome because synchrony breaks down during the latter part of replication (O'Sullivan and Sueoka, 1967). One of the reasons for the deterioration of synchrony may be the toxic effect of 5-BUDR (Laird and Bodmer, 1967). In the experiments labeling the terminus of the chromosome spores were germinated in TdR medium and then allowed to continue replication in the presence of PEA and 5-BUDR. If the chromosomes had completed replication then the met marker would have been hybridly labeled. None of the DNA peaks as measured by O.D. were situated at the light or hybrid densities of 1.703 and 1.742 g/ml, but were located at intermediate densities between the light and hybrid densities. The heaviest DNA peaks came from cells exposed to 5-BUDR the longest time, while the lightest DNA peaks were from cells exposed to 5-BUDR the shortest time. If the intermediate density was due to exchange of DNA-TdR for 5-BUDR in the

medium, then when the DNA molecules were broken into smaller pieces by shearing, the density of the DNA molecules should remain the same. When these DNA samples were sheared through a syringe needle and fractionated by CsCl density gradient centrifugation, most of the DNA molecules appeared at the light density as evidenced by the increased O.D. This result is consistent with the hypothesis that if no replication point exists in the chromosome and the DNA is isolated with minimum shear, then the labeled DNA would not separate from the unlabeled DNA. Shearing of these chromosomes into smaller molecules should allow separation of the labeled and unlabeled DNA in CsCl density gradient centrifugation. The facts that PEA did not inhibit dichotomous replication and that the chromosomes did not complete replication in the presence of 5-BuDR interfered with the interpretation of the genetic results of this experiment.

## SUMMARY

The existence or nonexistence of protein linkers in the bacterial chromosome was studied. DNA was isolated from B. subtilis by a procedure which attempts to eliminate the action of degrading nucleases and the degrading effects of mechanical shear. In this procedure most of the protein is removed. No genetic separation could be detected when sheared and unsheared DNA samples were fractionated on MAK columns. There was a difference in the elution pattern between the sheared and unsheared DNA samples; the sheared DNA sample showed a sharp elution peak while the unsheared sample showed a very broad elution pattern.

The question of whether the replication point is a weak point in the chromosome of B. subtilis was studied in experiments similar to the density transfer experiments of Yoshikawa and Sueoka (1963b). Two sets of experiments were performed: (1) experiments labeling the origin of the chromosome and (2) experiments labeling the terminus of the chromosome. The DNA samples labeled with 5-BUDR were centrifuged in a CsCl density gradient, and the fractions were assayed for genetic activity and DNA concentration. The results of these experiments offer support for the hypothesis that the replication point is a weak point in the chromosome because:

- (1) labeled markers appeared at the hybrid densities when a replication point existed in the chromosome;
- (2) when conditions were created to obtain partly labeled chromosomes with no replication points:

- (a) the majority of DNA molecules appeared at an intermediate density between the hybrid and the light densities,
- (b) the longer the cells were exposed to 5-BUdR, the more densely located was the DNA isolated from these cells,
- (c) the labeled part of the chromosome did not separate into the hybrid peak unless dichotomous replication had occurred, and
- (d) after shearing, the DNA molecules appeared mainly at the light density.

The facts that PEA did not inhibit dichotomous replication and chromosomes did not complete replication in the presence of 5-BUdR in these experiments interfered with the interpretation of the genetic results.

In the studies of the effects of PEA on the viability of B. subtilis it was found that concentrations of PEA slightly above the bacteriostatic concentration lysed actively growing cells of B. subtilis. When actively growing cell cultures of S. aureus and E. coli were examined, lysis was caused by PEA.

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